Attachments #8

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 February 2002 (21.02.2002)

PCT

(10) International Publication Number
**WO 02/13606 A2**

(51) International Patent Classification[7]: A01N

(21) International Application Number: PCT/US01/25543

(22) International Filing Date: 14 August 2001 (14.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/639,576 14 August 2000 (14.08.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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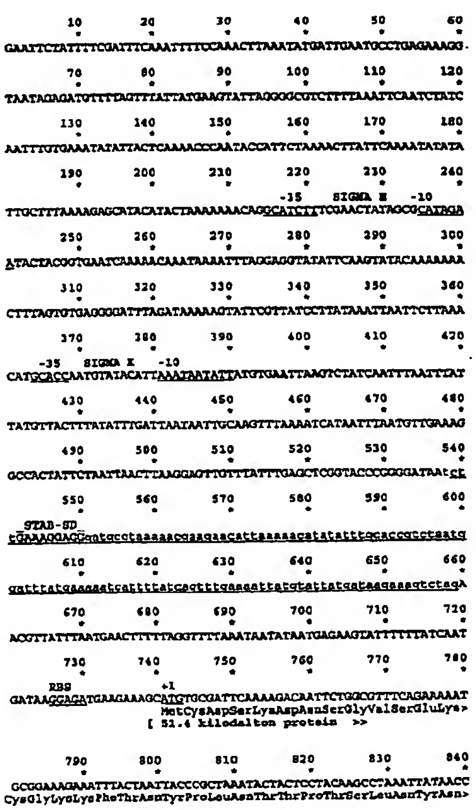
(54) Title: IMPROVED INSECTICIDAL BACTERIA, AND METHODS FOR MAKING AND USING THEM



**(57) Abstract:** The invention relates to nucleic acid sequences comprising a BtI or BtII promoter, or a combination of a BtI and a BtII promoter, at least 6 contiguous nucleotides of a bacterial STAB-SD sequence, and a sequence encoding a polypeptide with at least 80% sequence identity to a 41.9 kD protein of the B. sphaericus ("Bs") binary toxin, which polypeptide has at least 50% of the toxicity of the native 41.9 kD protein. Optionally, the sequence further encodes a second polypeptide with at least 80% sequence identity to the 51.4 kD protein of the Bs binary toxin and which can function as a binding domain for the Bs 41.9 kD toxin protein. When the nucleic acid sequences are expressed in B. thuringiensis ("Bt") or Bs cells, they result in at least 10 times the production of Bs binary toxin compared to untransformed Bs cells. The invention provides nucleic acid sequences, expression vectors, host cells, and methods of increasing the toxicity of an insecticidal bacterium by transforming the bacterium with nucleic acid sequences of the invention. Further, the invention relates to the discovery that the Cyt1Aa1 protein of Bt subspecies israelensis ("Bti") reverses resistance to Bs binary toxin in larvae of Bs-resistant mosquitoes. The invention provides Bs cells expressing Bti Cyt1Aa1 protein, and methods of reducing resistance to Bs binary toxin by co-administering the Cyt1Aa1 protein with Bs binary toxin.

WO 02/13606 A2

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# IMPROVED INSECTICIDAL BACTERIA, AND METHODS FOR MAKING AND USING THEM

## CROSS-REFERENCES TO RELATED APPLICATIONS

[01] This application claims priority from U.S. Patent Application No. 09/639,576, filed August 14, 2000, the contents of which are incorporated by reference..

## STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[02] Not applicable.

## BACKGROUND OF THE INVENTION

[03] Despite advances in medical science and new drugs, malaria, filariasis, dengue and the viral encephalilitides remain important diseases of humans, with an estimated 2 billion people worldwide living in areas where these are endemic (*The World Health Report - 1999*, World Health Organization, Geneva, Switzerland (1999)) The causative agents of these diseases are transmitted by mosquitoes, and therefore disease control methods have relied heavily on broad spectrum chemical insecticides to reduce mosquito populations. However, chemical insecticide usage is being phased out in many countries due the development of insecticide resistance in mosquito populations. Furthermore, many governments restrict use of these chemicals because of concerns over their effects on the environment, especially on non-target beneficial insects, and vertebrates through contamination of food and water supplies.

[04] As a result of these problems, the World Health Organization is facilitating the replacement of chemical with bacterial-based insecticides through the development of standards for their registration and use (Guideline specifications for bacterial larvicides for public health use, WHO Document WHO/CDS/CPC/WHOPES/99.2, World Health Organization, Geneva, Switzerland (1999)). Some of the products based on bacteria are designed to control mosquito larvae, the two most widely used of which are Vectobac® and Teknar®, both of which are based on *Bacillus thuringiensis* subsp. *israelensis*. In addition, Vectolex®, a new product based on *B. sphaericus* has come to market recently for control of the mosquito vectors of filariasis and viral diseases. These products have achieved moderate commercial success, but their high cost and lower efficacy compared to many

STAB-SD sequence are a 9-nucleotide bacterial STAB-SD sequence. In some preferred embodiments, the bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6). The *B. thuringiensis* promoter is a *cry* promoter, and in particular can be a *cryI* promoter. Further, the *B. thuringiensis* promoter can be *cry1Aa1, cry1Aa2, cry1Aa3, cry1Aa4, cry1Aa5, cry1Aa6, cry1Ba1, cry1Ba2, cry1Ca1, cry1Ca2, cry1Ca3, cry1Ca4, cry1Ca5, cry1Ca6, cry1Ca7, cry1Fa1, cry1Fa2, cyt1Aa1, cyt1Aa2, cyt1Aa3,* or *cyt1Aa4*. In some preferred embodiments, the *B. thuringiensis* promoter is a *cyt1Aa1* promoter. The nucleic acid can have both a BtI promoter and a BtII promoter, and the two promoters can be overlapping.

[11] The invention further provides expression vectors comprising the nucleic acids described above, and host cells comprising the expression vectors. The host cells can further comprise a 20 kD protein encoded by the Bti *cry11A* operon. In preferred embodiments, the host cell is a *B. thuringiensis* cell or a *B. sphaericus* cell.

[12] The invention further provides a nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter which binds a sigma factor A protein, 6 or more contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD toxin protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9. In more preferred embodiments, the first polypeptide has at least 90% sequence identity to SEQ ID NO:9 and in the most preferred embodiments, the first polypeptide has the sequence of SEQ ID NO.:9. The sequence encoding the first polypeptide can further comprise a sequence encoding a second polypeptide with at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), which polypeptide functions as a binding domain for the 41.9 kD toxin protein of SEQ ID NO:9. In preferred embodiments, the second polypeptide has at least 90% sequence identity to SEQ ID NO:8 and in the most preferred embodiment, has the sequence of SEQ ID NO:8. The 6 or more contiguous nucleotides of a bacterial STAB-SD sequence can be 6, 7, or 8 contiguous nucleotides of a 9-nucleotide bacterial STAB-SD sequence. In preferred embodiments, the 6 or more contiguous nucleotides of a bacterial STAB-SD sequence are a 9-nucleotide bacterial STAB-SD sequence. In some preferred embodiments, the bacterial STAB-SD sequence is selected from

the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6). In some embodiments, the nucleic acids further comprise a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence. In particularly preferred embodiments, the two sequences are both 9-mer bacterial STAB-SD sequences and, in especially preferred embodiments, the STAB-SD sequences are 30-40 nucelotides apart.

[13] The invention further provides methods of enhancing production of *B. sphaericus* binary toxin in a host bacterial cell, said method comprising: (a) transforming the host cell with a nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, at least 6 contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and (b) expressing said nucleic acid sequence in the host cell; whereby expression of said nucleic acid sequence enhances production of *B. sphaericus* binary toxin as compared to production of *B. sphaericus* binary toxin in a wild-type *B. sphaericus* cell that is not transformed with said nucleic acid sequence. In preferred embodiments, the first polypeptide has at least 90% sequence identity to SEQ ID NO:9. In particularly preferred embodiments, the first polypeptide has the sequence of SEQ ID NO.:9. In some embodiments, the sequence encoding said first polypeptide further encodes a second polypeptide with at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), which polypeptide functions as a binding domain for the 41.9 kD toxin protein of SEQ ID NO:9. In preferred embodiments, the second polypeptide has at least 90% sequence identity to SEQ ID NO:8, and in particularly preferred embodiments, the second polypeptide has the sequence of SEQ ID NO:8. In some embodiments, the nucleic acids further comprise a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence. In particularly preferred embodiments, the two sequences are both 9-mer bacterial STAB-SD sequences and, in especially preferred embodiments, the STAB-SD sequences are 30-40 nucelotides apart.

[14] In preferred embodiments, the 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence. In particularly preferred embodiments, the bacterial STAB-SD sequence is selected from the group

consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6). Preferably, the host cell is a *B. thuringiensis* cell or a *B. sphaericus* cell. In preferred embodiments, the host bacterial cell further expresses a 20 kD product of a *cry11A* gene.

[15] In another group of embodiments, the invention provides methods of creating a recombinant bacterium, said method comprising the steps of: (a) transforming the recombinant bacterium with a nucleic acid sequence comprising, in the following order: a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, at least 6 contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and (b) expressing the nucleic acid sequence in the host cell. In preferred embodiments, the first polypeptide has at least 90% sequence identity to SEQ ID NO:9, and in particularly preferred embodiments, has the sequence of SEQ ID NO.:9. The sequence encoding the first polypeptide can further comprise a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for the 41.9 kD toxin protein of SEQ ID NO:9. In preferred embodiments, the second polypeptide has at least 90% sequence identity to SEQ ID NO:8, and in particularly preferred embodiments, has the sequence of SEQ ID NO:8. In some embodiments, the 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence. In more preferred embodiments, the bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6). In some embodiments, the nucleic acids further comprise a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence. In particularly preferred embodiments, the two sequences are both 9-mer bacterial STAB-SD sequences and, in especially preferred embodiments, the STAB-SD sequences are 30-40 nucelotides apart. In preferred embodiments, the recombinant bacterium is selected from the group consisting of *B. thuringiensis, B. sphaericus*, and a member of a *Bacillus* species other than Bti or Bs.

[16] In yet another group of embodiments, the invention provides a method of increasing toxicity of a *B. thuringiensis* bacterium to a larva of a mosquito, said method comprising the steps of: (a) transforming said bacterium with a nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, 6 or more contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and (b) expressing said nucleic acid sequence in the bacterium; whereby expression of said nucleic acid sequence renders said bacterium more toxic to said larva than a wild-type *B. sphaericus* cell that is not transformed with said nucleic acid sequence. In preferred embodiments, the first polypeptide has at least 90% sequence identity to SEQ ID NO:9. In particularly preferred embodiments, the first polypeptide has the sequence of SEQ ID NO:9. In some embodiments, the nucleic acid sequence further comprises a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence. In particularly preferred embodiments, the two sequences of at least 6 nucleotides are both 9-mer bacterial STAB-SD sequences and, in especially preferred embodiments, the STAB-SD sequences are 30-40 nucelotides apart.

[17] In preferred embodiments, the sequence encoding the first polypeptide further comprises a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for a 41.9 kD toxin protein of SEQ ID NO:9. In more preferred embodiments, the second polypeptide has at least 90% sequence identity to SEQ ID NO:8 and in particularly preferred embodiments, the second polypeptide has the sequence of SEQ ID NO:8. Additionally, in preferred embodiments, said 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence and, in particularly preferred embodiments, the 9-nucleotide bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6). In especially preferred embodiments, the bacterium further comprises a 20 kD product of the *cry11A* gene.

[18] In yet another group of embodiments, the invention provides recombinant cells of *B. sphaericus*, said cells comprising nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter selected from the group consisting of a

BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, at least 6 contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9. In preferred embodiments, the first polypeptide has at least 90% sequence identity to SEQ ID NO:9 and in more preferred embodiments, has the sequence SEQ ID NO.:9. In some embodiments, the nucleic acids further comprise a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence. In particularly preferred embodiments, the two at least 6 nucleotide sequences are both 9-mer bacterial STAB-SD sequences and, in especially preferred embodiments, the STAB-SD sequences are 30-40 nucelotides apart.

[19] In preferred embodiments, the sequence encoding the first polypeptide further comprises a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for a 41.9 kD toxin protein of SEQ ID NO:9. In more preferred embodiments, the second polypeptide has at least 90% sequence identity to SEQ ID NO:8 and in especially preferred embodiments, has the sequence of SEQ ID NO:8. In some embodiments, the 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence and, in preferred embodiments, the 9-nucleotide bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6). In some preferred embodiments, the *B. thuringiensis* promoter is a *cry* promoter. In more preferred embodiments, the *B. thuringiensis* promoter is selected from the group consisting of *cry1Aa1, cry1Aa2, cry1Aa3, cry1Aa4, cry1Aa5, cry1Aa6, cry1Ba1, cry1Ba2, cry1Ca1, cry1Ca2, cry1Ca3, cry1Ca4, cry1Ca5, cry1Ca6, cry1Ca7, cry1Fa1, cry1Fa2, cyt1Aa1, cyt1Aa2, cyt1Aa3, and cyt1Aa4* and, in particularly preferred embodiments, is a *cyt1Aa1* promoter. In particularly preferred embodiments, the cell further expresses a 20 kD product of a *cry11A* operon.

[20] Further, the invention provides methods for increasing toxicity of a *B. sphaericus* cell, said methods comprising (a) transforming the cell with a nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, at least 6 contiguous nucleotide of a bacterial STAB-SD sequence, a ribosome

binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and (b) expressing said nucleic acid sequence in the host cell; whereby expression of said nucleic acid sequence increases toxicity of said cell compared a wild-type *B. sphaericus* cell that is not transformed with said nucleic acid sequence. In preferred embodiments, the first polypeptide has at least 90% sequence identity to SEQ ID NO:8 and, in particularly preferred embodiments, has the sequence of SEQ ID NO.:9. In preferred embodiments, the sequence encoding said first polypeptide further comprises a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for a 41.9 kD toxin protein of SEQ ID NO:9. In more preferred embodiments, the second polypeptide has at least 90% sequence identity to SEQ ID NO:8 and, in particularly preferred embodiments, has the sequence of SEQ ID NO:8. In some embodiments, the nucleic acid sequence further comprises a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence. In particularly preferred embodiments, the two at least 6 nucleotide sequences are both 9-mer bacterial STAB-SD sequences and, in especially preferred embodiments, the STAB-SD sequences are 30-40 nucelotides apart.

**[21]** In preferred embodiments, the 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence and, in particularly preferred embodiments, the 9-nucleotide bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6). In preferred embodiments of the methods, the *B. thuringiensis* promoter is a *cry* promoter. In more preferred methods, the *B. thuringiensis* promoter is a selected from the group consisting of *cry1Aa1, cry1Aa2, cry1Aa3, cry1Aa4, cry1Aa5, cry1Aa6, cry1Ba1, cry1Ba2, cry1Ca1, cry1Ca2, cry1Ca3, cry1Ca4, cry1Ca5, cry1Ca6, cry1Ca7, cry1Fa1*, cry1Fa2, *cyt1Aa1, cyt1Aa2, cyt1Aa3, and cyt1Aa4.* In the most preferred method, the *B. thuringiensis* promoter is a *cyt1Aa1* promoter.

**[22]** In yet another group of embodiments, the invention provides methods for reducing resistance to a *B. sphaericus* binary toxin, said methods comprising expressing a *B. thuringiensis* subsp. *israelensis* ("Bti") Cyt1Aa1 protein in a *B. sphaericus* cell expressing said binary toxin.

[23] The invention also provides methods for reducing resistance to a *B. sphaericus* binary toxin, said method comprising expressing a Bti Cyt1Aa1 protein in a *B. thuringiensis* cell expressing said binary toxin and for reducing resistance to a *B. sphaericus* binary toxin, said method comprising administering Bti Cyt1Aa1 protein with said binary toxin. The Bti Cyt1Aa1 protein can be in a powder of lysed, lyophilized Bti cell; it can also be a purified protein. The Bti Cyt1Aa1 protein can be administered in a Cyt1Aa1 protein to Bs ratio selected from about 1:2 to about 1:50. Preferably, the Bti Cyt1Aa1 protein is administered in a Cyt1Aa1 protein to Bs ratio of about 1:10.

BRIEF DESCRIPTION OF THE DRAWINGS

[24] **Figure 1** sets forth the nucleotide sequence (SEQ ID NO:7) and encoded amino acid sequence of a fragment used to clone Bs binary toxin into a plasmid. "Sigma E" and Sigma K" denote the binding sites for sigma factors E and K, respectively. The underlined sequence between nucleotides 537 and 660 denotes a portion cloned into the sequence by PCR to introduce a STAB-SD sequence, which is denoted both by capital letters in the underlined portion, and the "STAB-SD superscript. The start and stop codons of the 51.4 kD protein (SEQ ID NO:8) and of the 41.9 kD protein (SEQ ID NO:9) of Bs binary toxin are noted under the amino acid sequence. The underlined sections between nucleotides 725 and 730, and between 2245 and 2250, marked "RBS," represent the ribosome binding sites.

DETAILED DESCRIPTION

**I. INTRODUCTION**

[25] The inventions provides nucleic acid sequences, vectors, host cells, and methods for obtaining high levels of synthesis of the binary toxin of *Bacillus sphaericus* ("Bs") in recombinant bacterial cells. Bs toxin is very potent, but is produced by wild-type Bs cells at low levels. This, coupled with it being only a single toxin (in contrast, for example, to Bt, which produces a complex of toxins), permits the rapid development of insects resistant to the toxin. Increasing the amount of toxin produced per cell increases the killing power of the resultant biopesticide formulation and decreases the possibility that larvae ingesting the biopesticide will survive. Moreover, the increase in amount of toxin per cell greatly increases the efficiency of the bacterial toxin fermentation production process, and reduces the amount of bacterial product that must be applied to achieve insect control. Thus, the invention markedly reduces the cost of production and use and makes biopesticides

more competitive with chemical pesticides, which can be effective, but more environmentally damaging.

[26] The nucleic acids of the invention are heterologous sequences made by inserting a STAB-SD nucleic acid sequence between a strong promoter from a Bt gene and the ribosome binding site, and combining this construct with a nucleic acid sequence encoding the binding protein of the Bs toxin, or the toxin protein, or both. In preferred embodiments, both proteins are present. Surprisingly, coupling a strong Bt promoter with the STAB-SD nucleic acid sequence results in a dramatic increase in the production of the Bs toxin, by at least 10, and usually 15 to 20, times over the amount of protein produced by standard strains of unaltered (wild-type) *B. sphaericus*. The presence of the Bs binary toxin, in turn, results in surprising increases in the toxicity of the recombinant cells. For example, the toxicity of recombinant cells against the larvae of mosquitoes of the genus *Culex* is increased by more than 10 fold compared to non-recombinant cells.

[27] Optionally, the recombinant cell further contains a 20 kD chaperone-like product of a *cry11A* operon. Surprisingly, the presence of this protein increases the synthesis of Bs protein from the nucleic acid sequence described above by an additional 50% to 100%, and thus increases production of the Bs toxin to some 20 to 30 times more than that produced by standard strains of Bs.

[28] Due to the costs of obtaining regulatory approval for new pesticides and the like, it is generally desirable that the toxicity of the bacterial cells be increased by at least about 5 times against an organism of interest to warrant investment. Efforts by others for more than a decade to produce Bs toxin in Bs and *Bacillus thuringiensis* ("Bt") have resulted in increases in amounts of toxin production of 2, 3, or 4 -fold, too modest to be of interest for commercial production or for field use. Thus, the ability of the invention to permit production of Bs toxin in amounts that are at least 10, more usually 15, and as much as 20, 25 or even 30 times as high as that produced in standard strains of Bs is a significant and surprising advance in the art. Equally surprisingly, in tests against *Culex* mosquitoes, a significant vector of human disease, the toxicity of Bt cells transformed with the nucleic acids of the invention was improved by at least 10 fold, without diminishing the toxicity of the cells to other genera of mosquitoes.

[29] Biopesticides such as Bt are produced commercially in bioreactors. The ability provided by the invention to increase the toxicity of bacterial cells such as Bt or Bs means the amount of toxin produced per unit of culture medium will be increased, permitting the culturing of smaller quantities, and a commensurately decrease in the of raw

materials used for the culture medium. Thus, the invention reduces the cost of producing biopesticides, which will extend the situations in which it is cost-effective to use them in place of chemical pesticides. Moreover, the invention also provides the ability to confer Bs toxin-based toxicity on normally non-toxic bacterial species, and especially on species of bacillus which are normally non-toxic to insect larvae. Since the attributes of these other bacterial species, such as persistence in particular environments, are likely to be different than of the Bt and Bs which thus far have served as biopesticides, the invention also provides biopesticides with a different range of attributes than those currently available. The invention thereby expands the range of options for public health officials and agricultural scientists in combating insect pests.

**[30]** The ability to produce high levels of Bs toxin is particularly useful to increase the toxicity of Bt subspecies, such as subsp. *israelensis*, which is useful to control dipteran pests such as mosquito and blackfly larvae (this strain of Bt is hereafter referred to as "Bti"), subsp. *kurstaki*, which is currently useful in controlling caterpillar pests, including, e.g., the corn earworm (*Heliothis zia*), the cabbage looper (*Trichoplusia ni*), and the fall army worm (*Spodoptera frugiperda*), and subsp. *morrisoni*, which is active, e.g., against coleopteran pests such as the Colorado potato beetle (*Leptinotarsa decemlineata*), and the cottonwood leaf beetle (*Chrysomela scripta*), as well as subsp. *tenebrionis*, and subsp. *aizawai*.

**[31]** Moreover, the invention permits the extension of the host range of the biopesticide (that is, it extends the organisms against which the biopesticide is toxic). Bs toxin is toxic primarily to larvae of *Culex* and *Anopheles* species, while Bti is more active against *Culex* and *Aedes* species. Thus, the expression of high levels of Bs toxin in Bti cells not only increases their toxicity to *Culex*, but also renders the cells more useful agents against *Anopheles* species. Since *Anopheles* species are a major vector of malaria, this increased host range alone makes the invention a major addition to the public health arsenal.

**[32]** The invention further relates to the discovery that the Cyt1Aa1 (also known as "Cyt1A") protein of Bti can restore toxicity of Bs to mosquitoes that were highly resistant to Bs toxin. Other groups have previously shown that the mechanism of resistance to Bs is a loss of binding to receptors in the insect midgut. Without wishing to be bound by theory, it appears that Cyt1Aa1 allows insertion of the Bs toxin into the midgut microvillar membrane, restoring toxicity.

[33] Working with a *Culex quinquefasciatus* population at least 30,000-fold resistant to *B. sphaericus* 2362, the strain used in commercial biopesticide formulations, combining Bti Cyt1Aa1 with *B. sphaericus* completely suppressed resistance. Some suppression of resistance has previously been shown with a different Cyt protein, Cyt1Ab from *B. thuringiensis* subsp. *medellin* ("Btm"). Thiery et al., Appl. Environ. Microbiol. 64: 3910-3916 (1998). Surprisingly, however, the suppression of resistance by Bti Cyt1A is several fold higher than that which was achieved with Btm Cyt1Ab. Moreover, since Bs 2362 is the strain commercially used and to which target mosquito populations have already developed significant resistance, it is particularly important to suppress resistance to this strain. Thus, the discovery that Bti Cyt1Aa1 protein restores toxicity to Bs 2362 offers a solution to a major problem which has discouraged the continued use of Bs as a biopesticide.

[34] This discovery can be exploited by producing Bti Cyt1Aa1 in a bacterial cell producing Bs toxin, such as a Bs cell or a Bt cell recombinantly altered to produce Bs toxin. If the Bs toxin is produced in a Bs cell recombinantly altered to express Bti Cyt1Aa1, it is preferred to also transform the cell to express the 20 kD chaperone-like protein encoded by the *cry11A* operon. The sequence of *cyt1Aa1* is available from GenBank under accession number X03182, and was published by Waalwijk et al., Nucl. Acids Res. 13:8207-8217 (1985). The *cry11A* operon and the encoded 20 kD protein are discussed further below.

[35] Alternatively, Bti Cyt1Aa protein or Bti cells producing Cyt1Aa (or cells of other Bacillus species recombinantly altered to produce Bti Cyt1Aa) can be added to cells, granules or powder produced from Bs to render the granules toxic to organisms which would otherwise be resistant.

[36] As shown in the Examples, below, relatively modest amounts of Cyt1Aa1 protein are sufficient to dramatically suppress or even to eliminate resistance. In preferred embodiments, the Cyt1Aa1 protein can be added to a Bs mixture in a ratio selected from 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 or 1:10, with 1:10 being the most preferred since it affords striking reversal of resistance with relatively low amounts of added material. Higher ratios, such as 1:12, 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, or 1:70 can also be employed if, for example, it is desired to reduce the cost of adding Cyt1Aa1 protein, with the understanding that lower ratios may provide somewhat lower suppression of resistance. The assays taught in Example 5 can be used to test any particular ratio to discern if it would provide the degree of reversal of resistance desired. Ratios of Cyt1Aa1 to Bs of less than 1:100 are not preferred.

[37] The Cyt1Aa protein can be added as purified granules; however, it is usually easier to add Cyt1Aa in the form of Cyt1Aa-producing Bti cells. Conveniently, the Bti cells are lysed and lyophilized to form a powder prior to mixing with the Bs. In preferred embodiments, the Bs is strain 2362.

[38] Based on our results, other Cyt1 proteins from Bti will work in the same manner to reverse resistance to Bs binary toxin.

[39] Persons of skill in the art are aware that Bs and Bt cells are generally not administered together. Without wishing to be bound by theory, this may be due to concerns that the weight of the spores produced by each species relative to the toxin may reduce the effective amount of toxin the target larvae can ingest. The modest amounts of Bti which need to be added to achieve suppression of resistance, however, remove this concern as a factor. The studies reported in the Examples show ample toxicity when Bti cells were mixed with Bs.

[40] Bt has been used commercially as a biopesticide for some 20 years, and Bs has been used commercially for some 5 years. The use of Bti and Bs in the field has been reviewed, for example, in Mulla, M. S., "Activity, field efficacy, and use of *Bacillus thuringiensis israelensis* against mosquitoes," pp. 134-160 and in Yap, H.-H., "Field trials of *Bacillus sphaericus* for mosquito control" pp. 307-320, *in* H. de Barjac and D. J. Sutherland. [eds.] *Bacterial control of mosquitoes and blackflies*. Rutgers University Press (New Brunswick, NJ, 1990). Persons of skill in the art are therefore familiar with growing large quantities of Bt and of Bs organisms, with formulating biopesticides from those organisms, and with applying the formulations in the field. The recombinant organisms and methods described herein can be used in any of the methods known in the art for formulating biopesticides from Bt and Bs cells.

[41] Recombinant Bs cells of the invention can be used in any of the methods in which Bs biopesticides are currently used, but can be applied at lower application rates proportionate to their increased toxicity compared to the strain currently used commercially. For example, if the recombinant Bs has a toxicity 10 times that of the current strain, then one-tenth the weight of the material currently used can be applied to obtain the same killing power. Moreover, recombinant cells which express Cyt1Aa1 can be used against mosquito populations which have become resistant to wild-type Bs binary toxin.

[42] Recombinant Bt cells of the invention producing Bs binary toxin can be used to control the organisms normally controlled by Bt, and in addition can be used against *Anopheles* species. As discussed in connection with recombinant Bs, above,

recombinant Bt expressing high levels of Bs toxin can be applied at lower application rates proportionate to the increased toxicity of the recombinant to the target organism compared to the strain currently used commercially. Thus, the invention permits the use of less material. Since reducing the amount of Bt or Bs means that less Bt or Bs has to be grown, less raw material is needed to produce the same amount of killing power and thus the net cost of producing enough material to treat a given amount of area is decreased.

[43] The sections below define terms used in this specification. They then discuss Bt and Bs bacteria and their toxins, Bt promoters suitable for use in the invention, STAB-SD sequences, the assembly of nucleic acid sequences of the invention, and the 20 kD chaperone-like protein, as well as making and using the nucleic acids, vectors, host cells and bacteria of the invention.

## II. DEFINITIONS

[44] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* (2d ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[45] "*Bacillus thuringiensis,*" "*B. thuringiensis,*" and "Bt" refer to a gram positive soil bacterium characterized by its ability to produce crystalline inclusions during sporulation. The inclusions include insecticidal endotoxins. The inclusions comprise insecticidal proteins (sometimes referred to as "crystal proteins") encoded by genes carried on plasmids. The bacteria can be "cured" of the plasmid by growing them at raised temperatures, resulting in cells which do not produce the crystal proteins. Such bacteria are referred to as "acrystalliferous" or "crystal minus" cells.

[46] "*Bacillus sphaericus,*" or "Bs" refers to a gram positive soil bacterium which also produces a parasporal crystal of proteins toxic to certain insects.

[47] "Binary toxin" refers to the toxin produced by Bs. The toxin is comprised of two proteins, one of which serves as a binding moiety and one of which serves as the toxin. The two proteins are capable of associating in a solution to form a functional toxin. The nucleotide and amino acid sequences of the proteins are reported in Baumann et al., J. Bacteriol. 170:2045-2050 (1988). The 51.4 kD protein (SEQ ID NO:8) functions as the binding domain and the 41.9 kD protein (SEQ ID NO:9) functions as the toxin domain. As used herein, references to either of the proteins includes natural variants and such synthetic variants as do not reduce by more than 50% (a) with respect to the 41.9 kD toxin protein, the toxicity of the protein, or (b) with respect to the 51.4 kD binding protein, the ability of the protein to permit binding of the toxin protein. Specifically contemplated are synthetic variants in which conservative substitutions of amino acids are made, such as substituting a glutamic acid for an aspartic acid, or vice versa.

[48] "Cry" and "Cyt" refer to members of two families of proteins produced by *B. thuringiensis*. The nomenclature in the art has recently changed from referring to the various Cry proteins by Roman numerals (intended to denote the apparent ranges of organisms to which the proteins are toxic) to Arabic numerals; the Cyt proteins were also redesignated. A comprehensive table correlating the nomenclature of the older designations and the current designations for some 130 Cry and Cyt proteins is set forth in Crickmore et al., Microbiol. Mol. Biol. Rev. 62:807-813 (1998).

[49] The protein now termed "Cyt1Aa1" was sometimes previously referred to as "CytA" or "Cyt1A;" references herein to CytA or to Cyt1A refer to Cyt1Aa1.

[50] Following standard usage in the art, the use of the terms "Cry" or "Cyt" herein denote the protein, while the lowercase, italicized terms "*cry*" or "*cyt*" refer to the genes.

[51] A "promoter" is an array of nucleic acid control sequences, e.g., the *cry1Ac1* promoter from *B. thuringiensis*, that direct transcription of an associated polynucleotide, which may be a heterologous or native polynucleotide. A promoter includes nucleic acid sequences near the start site of transcription, such as a polymerase binding site. The promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

[52] "Sigma factors" refer to proteins known to recognize particular sequences in DNA and which form part of a complex of proteins which facilitate the initiation of transcription of the DNA by RNA polymerase. As is known in the art, the

sequence of the sigma factor proteins was determined from studies in *B. subtilis*, the proteins performing the same functions in other *Bacillus* species have about 80-95% sequence to the sigma factors of *B. subtilis*. Accordingly, the factors which in Bt perform the same role as the sigma factors of *B. subtilis* have slightly different sequences than those of the paradigm proteins of *B. subtilis*. The term "sigma factor" herein refers to proteins in Bt performing the same function as the sigma factors of *B. subtilis* and having about 80-95% or higher sequence homology to those proteins. To make the point that these proteins correspond, but are not necessarily identical in sequence to the *B. subtilis* proteins, they are also sometimes referred to herein as "sigma-like" factors or proteins.

[53] A "BtI" promoter" refers to a promoter which is recognized by sigma factor -E. A "BtII promoter" refers to a promoter which is recognized by sigma factor K.

[54] A "strong BtI or BtII promoter" refers to a promoter which, when operably linked to a nucleic acid sequence encoding a protein, and expressed in a Bt cell, results in the protein comprising at least 5%, more preferably 10%, and most preferably 15% or more of the dry weight of the cell.

[55] "Polynucleotide" and "nucleic acid" refer to a polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Thus, the term includes nucleotide polymers in which the nucleotides and the linkages between them include non-naturally occurring synthetic analogs. It will be understood that, where required by context, when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

[56] "Recombinant" refers to polynucleotides synthesized or otherwise manipulated *in vitro* ("recombinant polynucleotides") and to methods of using recombinant polynucleotides to produce gene products encoded by those polynucleotides in cells or other biological systems. For example, an cloned polynucleotide may be inserted into a suitable expression vector, such as a bacterial plasmid, and the plasmid can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell" or a "recombinant bacterium." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant protein." A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

[57] A "heterologous polynucleotide sequence" or a "heterologous nucleic acid" is a relative term referring to a polynucleotide that is functionally related to another polynucleotide, such as a promoter sequence, in a manner so that the two polynucleotide sequences are not arranged in the same relationship to each other as in nature. Heterologous polynucleotide sequences include, e.g., a promoter operably linked to a heterologous nucleic acid, and a polynucleotide including its native promoter that is inserted into a heterologous vector for transformation into a recombinant host cell. Heterologous polynucleotide sequences are considered "exogenous" because they are introduced to the host cell via transformation techniques. However, the heterologous polynucleotide can originate from a foreign source or from the same source. Modification of the heterologous polynucleotide sequence may occur, e.g., by treating the polynucleotide with a restriction enzyme to generate a polynucleotide sequence that can be operably linked to a regulatory element. Modification can also occur by techniques such as site-directed mutagenesis.

[58] The term "expressed endogenously" refers to polynucleotides that are native to the host cell and are naturally expressed in the host cell.

[59] An "expression cassette" refers to a series of polynucleotide elements that permit transcription of a gene in a host cell. Typically, the expression cassette includes a promoter and a heterologous or native polynucleotide sequence that is transcribed. Expression cassettes may also include, e.g., transcription termination signals, polyadenylation signals, and enhancer elements.

[60] The term "operably linked" refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the sequence). Thus, a polynucleotide is "operably linked to a promoter" when there is a functional linkage between a polynucleotide expression control sequence (such as a promoter or other transcription regulation sequences) and a second polynucleotide sequence (e.g., a native or a heterologous polynucleotide), where the expression control sequence directs transcription of the polynucleotide.

[61] An "insecticidal endotoxin" refers to a family of genes encoding endotoxin proteins that exhibit insecticidal activity, also known as crystal proteins, e.g., Cry2A, Cry3A, Cry1B, Cry1C and Bs binary toxin (see Hofte & Whiteley, *Microbiol. Rev.* 53: 242-255 (1989)). Such insecticidal endotoxins are produced by *Bacillus thuringiensis* and are toxic to insects, particularly insect larvae.

[62] An "insecticidally effective amount" of an insecticidal endotoxin is a unit dose amount that provides insecticidal activity when applied to a plant, soil, or another "locus," e.g., site or location.

[63] The "gene encoding the *cry11A* operon 20 kDa protein" (20 kDa protein gene) refers to the gene in the *cry11A* operon that encodes a protein of approximately 20 kDa (as described in Frutos et al., *Biochem. Sys. and Ecol.* 19:599-609 (1991); see Frutos et al. Figure 4 for nucleotide and amino acid sequence).

[64] "Enhancing production" refers to an activity of a first protein, such as the *cry11A* operon 20 kDa protein, that increases the net amount of a second protein, such as an insecticidal endotoxin, in a host cell.

[65] "Competent to express" refers to a host cell that provides a sufficient cellular environment for expression of endogenous and/or exogenous polynucleotides.

[66] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

[67] The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

[68] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[69] Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer

Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally, Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) ("Ausubel")).

[70] Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410 and Altschuel *et al.* (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

[71] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino

acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[72] A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

[73] The terms "stringent hybridization conditions" or "stringent conditions" refer to conditions under which a nucleic acid sequence will hybridize to its complement, but not to other sequences in any significant degree. Stringent conditions in the context of nucleic acid hybridizations are sequence dependent and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology -- Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, New York, (1993) (the entirety of Tijssen is hereby incorporated by reference). Very stringent conditions are selected to be equal to the $T_M$ point for a particular probe. Less stringent conditions, by contrast, are those in which a nucleic acid sequence will bind to imperfectly matched sequences. Stringency can be controlled by changing temperature, salt concentration, the presence of organic compounds, such as formamide or DMSO, or all of these. The effects of changing these parameters are well known in the art. The effect on $T_m$ of changes in the concentration of formamide, for example, is reduced to the following equation: $T_m = 81.5 + 16.6 (\log Na^+) + 0.41 (\%G+C) - (600/\text{oligo length}) - 0.63(\%\text{formamide})$. Reductions in Tm due to TMAC and the effects of changing salt concentrations are also well known. Changes in the temperature are generally a preferred means of controlling stringency for convenience, ease of control, and reversibility.

[74] "Conservative substitution" refers to the substitution in a polypeptide of an amino acid with a functionally similar amino acid. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

*See also*, Creighton, *PROTEINS*, W.H. Freeman and Company, New York (1984).

## III. THE BTI AND BS TOXINS

[75] Bti and Bs are aerobic, gram positive sporeforming soil organisms which produce proteinaceous crystalline inclusions during sporulation. The crystals of Bt subspecies are toxic to the larvae of a wide variety of leipdopteran, coleopteran and dipteran species. The Introduction lists some of the insects against which different subspecies of Bt are currently used. Bt comprises about 90% of all biopesticides used. Agaisse and Lereclus. J. Bacteriol. 177:6027-6033 (1995).

[76] The *cry* and *cyt* genes encode the various insecticidal proteins produced by Bt. A large number of these genes have been identified and sequenced and are well known in the art. For example, Crickmore et al., Microbiol Mol Biol Rev., 62:807-813 (1998) (the whole of Crickmore et al. is hereby incorporated by reference) provide a table setting forth the GenBank accession numbers for the sequences of over 120 identified Bt *cry* genes and 9 Bt *cyt* genes.

[77] As might be expected from the name, the binary toxin of Bs is composed of two proteins, one of 51.4 kD and one of 41.9 kD. The nucleotide and amino acid sequences of the proteins have been known for over a decade and are reported in Baumann et al., J. Bacteriol. 170:2045-2050 (1988). The 51.4 kD protein (SEQ ID NO:8) functions as the binding domain and the 41.9 kD protein (SEQ ID NO:9) functions as the toxin domain, thus, equimolar quantities of both proteins should usually be present for maximal toxin function. Conveniently, this can be accomplished by having a nucleic acid sequence encoding both proteins downstream of the promoter-STAB-SD construct of the invention so that both proteins are expressed at the same time and in approximately the same amounts. If the nucleic acid sequence of only one of the two Bs toxin proteins is placed under the control of a promoter-STAB-SD sequence of the invention, it is desirable that a sequence encoding the other Bs toxin protein be placed under the transcriptional control of a like promoter construct so that the two proteins are produced in equimolar or roughly equimolar amounts.

[78] Persons of skill will appreciate that it is possible to make changes to the sequences of SEQ ID NO:8 and 9 and still have a protein which functions as does a Bs toxin using routine genetic engineering techniques. For example, conservative substitutions of amino acids can be made in the sequence of the Bs binary toxin protein (SEQ ID NO:9) to result in a protein or polypeptide that has high sequence identity and which retains at least a portion of the toxicity of the native protein, and similar alterations can be made in the Bs binary toxin binding protein (SEQ ID NO:8) while nonetheless permit the protein to function as a binding protein for the toxin protein. Preferably such proteins or polypeptides have at least about 75% identity to the native Bs 41.9 kD or 51.4 kD proteins, more preferably at 80%, still more preferably 85%, even more preferably 90% and most preferably 95% or greater identity to SEQ ID NO:8 or SEQ ID NO:9. The function of any particular polypeptide to serve in place of a native Bs toxin can be readily tested by assays known in the art and as set forth herein. It is preferable that polypeptides serving in place of the native 41.9 kD toxin protein (SEQ ID NO:9) have at least 50% of the toxicity of native Bs toxin, more preferably 60%, still more preferably 70% or more, even more preferably about 80% or more, yet more preferably, 90% or more of the toxicity of native Bs toxin. It is preferable that polypeptides serving in place of the native 51.4 kD binding protein (SEQ ID NO:8) of the Bs binary toxin can bind to insect receptors for that binding protein. Such binding can be determined for example, by immunohistochemistry (which is qualitative) or brush border membrane binding assays (these assays, which are also known as "brush border membrane vesicle binding assays," provide quantitative assessment of relative binding ability). Such assays are well known in the art.

[79] Surprisingly, we have found that the Cyt1A protein from Bt can restore toxicity of the toxin to larvae which have lost receptors for the binding protein of the Bs binary toxin. Accordingly, if desired, the Cyt1A protein can be co-expressed in a cell expressing the 41.9 kD toxin protein of the Bs binary toxin in place of some or all of the 51.4 kD binding domain protein and the proteins will be toxic to target larvae ingesting the cell or biopesticides made from the cell. Thus, for example, a Bt cell, such as Bti, can be transformed by introducing a nucleic acid sequence encoding the Cyt1A protein with a promoter that will express amounts roughly similar to the amount of Bs 41.9 kD protein. Alternatively, Cyt1A protein and Bs binary toxin can be separately produced and mixed to render Bs toxin-resistant mosquitoes sensitive to the toxin. In a variation of this use, Cyt1A protein can be added to 41.9 kD Bs toxin protein (that has been, for example, recombinantly expressed) and the mixture used against mosquito populations resistant to Bs toxin. Because

the mechanism of action may not be the same as that of the 51.4 kD binding domain protein, it appears that the amount of the Cyt1A protein need not be as closely matched to the amount of the toxin protein as would be true for the 51.4 kD Bs binding protein to achieve full toxic effect.

## IV. BT PROMOTERS

[80] The invention uses promoters from Bt *cry* or *cyt* genes to drive the expression of Bs toxin. These genes encode the various insecticidal proteins produced by Bt. As noted in the previous section, Table 1 of Crickmore et al., Microbiol Mol Biol Rev., *62:807-813 (1998) sets forth a convenient listing* of the names and GenBank accession numbers for the sequences of 120 Bt *cry* genes and 9 Bt *cyt* genes. Persons of skill in the art will appreciate that the 5' sequence preceding the start codon for the coding region described in the listing for each of these genes comprises the promoter region.

[81] As persons of skill are aware, gene transcription of Cry and Cyt proteins is temporally regulated by the presence of sequences to which proteins known as sigma factors bind. The *sigma factors recruit other proteins which form* a complex permitting the RNA polymerase to initiate transcription of the DNA. The promoters of the *cry* or *cyt* genes are generally classified into three categories, based on the sigma factors which bind to them. They are the sigma-E promoters, the sigma-K promoters, and the sigma-A promoters.

[82] The sigma-E promoters are also known as BtI promoters and sigma-K promoters are also known as BtII promoters (with regard to the discussion of promoters herein, the terms "sigma-E" and "BtI" are used interchangeably, as are the terms "sigma-K" and "BtII"). A number of *cry* genes are active during sporulation and are generally driven by BtI or BtII promoters, with BtI promoters active earlier in sporulation than are the BtII promoters. *See generally*, Agaisse and Lereclus, J. Bacteriol. 177:6027-6032 (1995) ("Agaisse and Lereclus 1995"). The sigma factors which recognize most *cry* genes are known. E.g., Agaisse and Lereclus 1995. For example, *cry4A* and *B* are recognized by BtI promoters.

[83] A number of *cry* genes have two promoters, one BtI and one BtII. The combination of these dual promoters serves to extend the expression of the gene over a longer period of the sporulation process. In some cases, the two promoters overlap. Non-sporulation dependent *cry* genes have yet another set of promoters, which are recognized by yet another sigma factor, sigma-A. *See*, e.g., Agaisse and Lereclus 1995.

[84] Any strong BtI or BtII promoter can be used in the nucleic acid sequences and methods of the invention. Dual BtI and BtII promoters, and particularly the overlapping BtI and BtII promoters, tend to be strong promoters of protein expression and are preferred forms of promoters for constructing nucleic acids of the invention. Members of the genes falling within the following groups which have dual promoters are especially preferred: *cry1A*, *cry1B*, *cry11A*, and *cyt1Aa*.

[85] For purposes of the invention, any BtI or BtII promoter which can drive expression of Cyt1Aa1 protein to comprise at least 5%, more preferably 10%, and most preferably 15% or more of the dry weight of an acrystalliferrous Bti cell, is considered a strong promoter and is an appropriate BtI or BtII promoter for purposes of the invention. Dual BtI and BtII promoters or promoter regions are preferred.

[86] In preferred embodiments, the BtI or BtII promoter is a *cry1* promoter or a *cyt1Aa1* (also known as a cyt1A) promoter. Due to the close phylogenetic relationship and high sequence identity of the *cry1* promoters (see, Crickmore et al., Microbiol. Mol. Biol. Rev. 62:807-813 (1998), any of the promoters of the genes designated in Crickmore et al. as a *cry1* gene is considered capable of driving high levels of expression of Bs binary toxin. Particularly preferred embodiments are *cry1Aa1* (formerly called *cry1A(a)*), *cry1Ba1* (formerly called *cry1B*), *cry1Ca1* (formerly called *cry1C*), and *cry1Fa1* (formerly called *cry1F*). It should be noted that each of these genes has other closely related genes. For example, *cry1Fa1* is closely related to *cry1Fa2*. The other members of the named *cry1* gene groups designated by the same capital letter and same lower case letter are considered to be almost as preferred as the first listed gene in the group (that is, *cry1Fa2* is almost as preferred as *cry1Fa1*).

[87] In another particularly preferred embodiment, the promoter can be the promoter from *cyt1Aa1*, which comprises both BtI and BtII promoters, and is accordingly sometimes referred to as a dual promoter. Since the promoter region contains two promoters, the promoter region of this gene is also termed the "*cyt1A* promoters." Based on phylogenetic analysis and sequence identity, the promoters of the other *cyt1Aa* genes are also sufficiently strong BtI or BtII or combined BtI and BtII promoters to be used in the compositions and methods of the invention.

[88] The non-sporulation dependent *cry* gene promoters are sigma-A promoters and are not generally satisfactory, except under a modified set of conditions. Non-sporulating forms of Bt, or course, do not divert their metabolic resources to spore production, and can accumulate toxin over a longer period than can sporulating forms. In

non-sporulating forms, therefore, sigma-A promoters can be used to accumulate high levels of toxin. Accordingly, BtI and BtII promoters are preferred for use in Bacillus, including Bt and Bs. In non-sporulating forms of Bacillus, sigma-A promoters may be used.

## V. STAB-SD SEQUENCES

[89] The crystal protein mRNAs of *B. thuringiensis* have an average half life of 10 minutes during sporulation, whereas the average half life of other mRNAs is between 1 to 2 minutes. This long half life may be responsible in part for the very high production of crystal proteins, which can be as much as 20-30% of the dry weight of the sporulated cells.

[90] The long half life of these proteins is related to two untranslated regions of the genes. First, there is a sequence in the 5' untranslated region, usually found between the promoter and the coding region, which is not involved in translation initiation but which is a determinant of stability for mRNA. The sequence is a consensus Shine-Dalgarno-("SD") like sequence. Second, the 3' terminal fragment of *cry* genes, such as *cry1Aa*, increases the half life of mRNA transcripts two to threefold. Agaisse and Lereclus, Mol. Microbiol., 20:633-643 (1996) (hereafter "Agaisse 1996").

[91] The 5' SD-like sequence appears to be involved in stabilizing the production of the proteins. It has, accordingly, been named a "STAB-SD" sequence. Agaisse 1996. Agaisse 1996 suggests that the STAB-SD is involved in interactions with the 3' end of 16S ribosomal RNA, and found that mutations of the STAB-SD sequence which were expected to abolish complementarity affected the stability conferred. Interestingly, the STAB-SD sequence of the protein then called cryIIIA (now called cry3A) showed putative interactions with the 3' end of *B. subtilis* 16S rRNA. Without wishing to be bound by theory, it appears that the polypurine (G and A) nucleotides of the STAB-SD sequence base-pair with a complementary sequence of pyrimidine nucleotides in the 3' end of the 16S rRNA subunit of the ribosome, and that this base pairing inhibits access of 5' endoribonuclease to the 5' end of the mRNA, increasing the half life of the message, and thereby enhancing the production of the encoded protein. Thus, it appears that STAB-SD sequences are not specific for particular species of bacteria, and that the STAB-SD sequences of other *Bacillus* species, and of other genera of bacteria, can be used to stabilize the production of proteins in *B. thuringiensis* and *B. sphaericus*.

[92] Agaisse 1996 reviewed databases and identified numerous examples of putative STAB-SD sequences in 5' untranslated regions ("UTR"), including those of four *cry*

genes from Bt, the *cwp* locus of *B. brevis*, and the *inlAB* locus of *Listeria monocytogenes*. Any of these STAB-SD sequences can be used to produce high levels of Bs toxin in *Bacillus* when placed between a strong *Bacillus* promoter and a ribosome binding site. The STAB-SD sequences identified share fairly high homology to one another.

[93] In preferred embodiments, the STAB-SD sequence is selected from the group consisting of GAAAGGAGG (the *cry3A* sequence, SEQ ID NO:1), GAAGGGGGG (the *cry3B* sequence, SEQ ID NO:2), GAGGGGGGG (the *cry3B2* sequence, SEQ ID NO:3), GAAAGGGGG (the *cry3D* sequence, SEQ ID NO:4), GAAAGGAGG (the *cwp* from *B. brevis* sequence, SEQ ID NO:5), and GAAAGGGGT (the *in1AB* from *L. monocytogenes*, SEQ ID NO:6). The *cry3A*, *cry3B*, *cry3B2* and *cry3D* sequences are particularly preferred. *Cry3A* is the most preferred embodiment. If desired, two or more STAB-SD sequences can be used in tandem. If multiple STAB-SD sequences are used, the sequences are preferably separated by about 30 to 40 nucleotides so that the STAB-SD sequences can interact with multiple 30S ribosomal subunits. The span length of the ribosomal subunit is about 30 nucleotides. The number of multiple STAB-SD sequences used should not be so great as to interfere with interaction between the STAB-SD sequences and the 3' end of *B. subtilis* 16SrRNA 3'. The presence of too many STAB-SD sequences can be determined by noting a decrease in protein production compared to a construct with one fewer STAB-SD sequence. One to two STAB-SD sequences are the most preferred. Similarly, while two STAB-SD sequences can be separated by any convenient distance (typically, 3 - 300 nucleotides), the effect of the separation can be tested by determining the production of protein by a construct with the separation being tested in comparison to a like construct with a separation of 33 to 40 nucleotides.

[94] Other sequences with high sequence identity to one of the STAB-SD sequences set forth above and which function as a STAB-SD sequence can be used in the nucleic acids and methods of the invention. The sequence should have at least 85% sequence identity to one of the STAB-SD sequences set forth above and should function to improve protein production. In preferred forms, the sequence has at least about 90% sequence identity, and even more preferably has about 95% or higher sequence identity. In addition, Agaisse 1996 provides guidance on changes to putative STAB-SD sequences which may deleteriously affect stability. In general, any change in a nucleotide which would abolish interaction between the STAB-SD sequence and the 3' end of *B. subtilis* 16SrRNA 3' is likely to reduce protein production and is not preferred.

[95] Surprisingly, it has also been discovered that the full STAB-SD sequence does not have to be used to enhance protein production. As few as 6 contiguous nucleotides of a STAB-SD sequence can function to improve stability. While not as powerful in stabilizing production of proteins as a full 9-nucleotide STAB-SD sequence, better results are obtained than if the protein is produced without the 6-mer (a sequence of a number of nucleotides or amino acids can be referred to in the art as the number and the suffix "-mer"). Thus, if desired, one of these STAB-SB subsequences can be used in place of a full STAB-SD sequence. Since, however, longer sequences bind more strongly to the corresponding pyrimidine sequence of the 16S rRNA ribosomal subunit, more preferably, 7 contiguous nucleotides of a STAB-SD sequence are used and, still more preferably, 8 contiguous nucleotides of a STAB-SD sequence are used. Use of a full-length 9 nucleotide STAB-SD sequence is the most preferred. Better results can also be achieved by using two of these constructs in tandem, such as two 6-mers. For ease of discussion in other sections of this specification, the term "STAB-SD sequence" includes a 6, 7, or 8 contiguous nucleotide subsequence of a STAB-SD sequence, unless otherwise required by context.

[96] Any putative nucleic acid sequence, or any desired modification to a known STAB-SD sequence, can be conveniently tested for its function as a STAB-SD protein by placing the sequence in a plasmid containing a galactosidase coding sequence following the assays and other methods taught in Agaisse 1996, or by substituting the sequence under consideration for the STAB-SD sequence in the procedure set forth in the Examples, below, and comparing the resulting protein production to the production of the same protein from the construct using the STAB-SD sequence set forth herein. Sequences which reduce production of Bs binary toxin to less than about 8 times that of wild-type Bs cells as measured by densiometric analysis of Coomassie blue-stained SDS-PAGE gels are less preferred.

## VI. ASSEMBLY OF NUCLEIC ACID SEQUENCES OF THE INVENTION

[97] Considerable information has developed in the art about the construction of promoters; in this context, the following discussion is offered to provide the specific information persons of skill may need to optimize placing a STAB-SD sequence between the sigma-factor binding site of a strong Bt promoter and the ribosome binding site.

[98] Figure 1 demonstrates an exemplary assembly of a nucleic acid sequence of the invention. In this embodiment, the *cyt1A* promoters comprise nucleotides 1-537. (Since the *cyt1A* gene contains two promoters, one a BtI promoter and the other a BtII promoter, the promoter region of the gene is sometimes referred to in the art as the "*cyt1A*

promoters.") The binding sites for the sigma E-like factor and the sigma K-like factor are shown with the notations "SIGMA E" and "SIGMA K," respectively, placed over the appropriate regions, with the terms "-35" and "-10" and underlined sequences designating the specific binding sites. The underlined nucleotides with the letters "RBS" at nucleotides 726 to 730 and 2246 to 2249 denote ribosome binding sites.

[99] The underlined nucleotides from 538 to 659 denote a sequence cloned in from the *cry3A* promoter. This sequence was cloned in to introduce the 9 nucleotide STAB-SD sequence; the longer sequence from the *cry3A* promoter was used because it is relatively more difficult to clone in a 9 nucleotide sequence. The sequence commencing at position 660 is a portion of the sequence of the binary toxin upstream of the coding region, followed by the coding region (as published by Baumann et al., J. Bacteriol. 170:2045-2050 (1988)). The particular portion upstream of the start codon at the position marked "+1" was selected simply because of the presence of a convenient restriction site. Shorter or longer portions could be used, and indeed, the entire promoter region of the binary toxin gene can be used if desired. In general, however, use of shorter sequences is preferred, not only for ease of manipulation but also to avoid the accidental inclusion of repressor sequences or the like which might happen to be present. Additionally, if a different sequence is used after the STAB-SD sequence, a ribosome binding site should be placed between the STAB-SD sequence and the start codon. Preferably, the ribosome binding site is positioned about 6 to about 10 nucleotides upstream of the start codon. The start and stop sites of the 51.4 kD and 41.9 kD Bs binary toxin proteins are also shown.

[100] The manner in which the elements of this exemplary sequence are joined can be varied substantially and still result in a sequence which works well in producing high levels of Bs binary toxin. In this sequence, some 121 nucleotides from the *cry3A* sequence were used to clone in the STAB-SD sequence. This was done simply for ease in cloning; the 9-nucleotide STAB-SD sequence (or, as explained in the preceding section, a 6, 7, or 8 contiguous nucleotide subsequence thereof) can be introduced by itself. For ease in cloning, however, it is usually preferable to use a sequence which encompasses the STAB-SD sequence and which is from about 20 to about 130 nucleotides in length. The STAB-SD sequence itself can be placed anywhere from about 10 bases downstream of the sigma factor binding site to just before an RBS sequence, which in turn should be about 6 to about 10 bases upstream of the start codon. That is, all or a portion of the promoter downstream of the sigma-factor binding site can be deleted, with the understanding that if the RBS of the promoter is deleted, another RBS, such as that from the Bs binary toxin, should

be placed about 6 to about 10 bases upstream of the start codon. As noted, in the sequence depicted in Figure 1, the sequence from position 660 on are from the native Bs binary toxin gene. Any particular sequence can be readily tested by substituting it in the assays taught in the Examples to determine whether it has a deleterious or advantageous effect on toxin production.

## VII. 20 KD CHAPERONE-LIKE PROTEIN

[101] In the methods of the present invention, host cells are transformed with a gene encoding a 20 kDa protein gene, which encodes a known protein (Frutos *et al.*, *supra*; Visick & Whitely, *supra*), to enhance the production of Bs binary toxin. The 20 kDa protein gene can be isolated and sequenced, for example, from two subspecies of *B. thuringiensis* (Frutos *et al.*, *Biochem. Syst. and Ecol.* 19: 599-609 (1991)). The level of expression of the 20 kDa protein has been characterized in cells transformed with the 20 kDa protein gene. Using methods and sequence information described herein and in International Patent Application WO97/39623, the 20 kDa protein gene can be isolated by those skilled in the art and used to construct recombinant expression vectors for transformation of a host cell.

[102] The host cells transformed with the 20 kD protein gene should be competent to express Bs binary toxin. The cells may express the Bs binary toxin, or the cells may be transformed with exogenous binary toxin expression vectors. As noted earlier, the sequence of both proteins of the Bs binary toxin is known. This sequence information can be used by one skilled in the art, along with the methods described herein, to construct recombinant vectors for transformation of a host cell, such as a Bs cell, with the gene encoding the 20 kD protein. Conveniently, the gene for the 20 kD protein can be placed on the same plasmid as the nucleic acid sequence for expressing high levels of Bs toxin.

## VIII. NUCLEIC ACIDS SEQUENCES AND VECTORS

[103] A recombinant expression vector for transformation of a host cell is prepared by first isolating the constituent polynucleotide sequences, as discussed herein. The polynucleotide sequences, e.g., a sequence encoding the Bs binary toxin driven by a promoter as discussed above, are then ligated to create a recombinant expression vector suitable for transformation of a host cell. Methods for isolating and preparing recombinant polynucleotides are well known to those skilled in the art. Sambrook *et al.*, *Molecular Cloning. A Laboratory Manual* (2d ed. 1989); Ausubel *et al.*, *Current Protocols in Molecular*

*Biology* (1995)), provide information sufficient to direct persons of skill through many cloning exercises.

[104] One preferred method for obtaining specific polynucleotides combines the use of synthetic oligonucleotide primers with polymerase extension or ligation on a mRNA or DNA template. Such a method, e.g., RT, PCR, or LCR, amplifies the desired nucleotide sequence (see U.S. Patents 4,683,195 and 4,683,202). Restriction endonuclease sites can be incorporated into the primers. Amplified polynucleotides are purified and ligated to form an expression cassette. Alterations in the natural gene sequence can be introduced by techniques such as *in vitro* mutagenesis and PCR using primers that have been designed to incorporate appropriate mutations. Another preferred method of isolating polynucleotide sequences uses known restriction endonuclease sites to isolate nucleic acid fragments from plasmids. The genes of interest can also be isolated by one of skill in the art using primers based on the known gene sequence.

[105] The isolated polynucleotide sequence of choice, e.g., the Bs binary toxin driven by the promoter sequence discussed above, is inserted into an "expression vector," "cloning vector," or "vector," terms which usually refer to plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors can replicate autonomously, or they can replicate by being inserted into the genome of the host cell. Often, it is desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in *B. thuringiensis* for expression. Additional elements of the vector can include, for example, selectable markers, e.g., tetracycline resistance or hygromycin resistance, which permit detection and/or selection of those cells transformed with the desired polynucleotide sequences (see, e.g., U.S. Patent 4,704,362). The particular vector used to transport the genetic information into the cell is also not particularly critical. Any suitable vector used for expression of recombinant proteins in host cells can be used. A preferred vector is pHT3101, which is an *E. coli-B. thuringiensis* shuttle vector (Lereclus *et al.*, *FEMS Microbiol. Lett.* 60: 211-218 (1989)).

[106] Expression vectors typically have an expression cassette that contains all the elements required for the expression of the polynucleotide of choice in a host cell. A typical expression cassette contains a promoter operably linked to the polynucleotide sequence of choice. The promoter used to direct expression of the Bs binary toxin is as described above, and is operably linked to a sequence encoding one or both of the Bs binary toxin proteins. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural

setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function. For expression of the 20 kD protein encoded by the *cry11A* operon, other promoters suitable for driving the expression of a heterologous gene in a host cell can be used, including those typically used in standard expression cassettes, *e.g.*, the β-galactosidase promoter. In one embodiment of the invention, the 20 kD protein gene is operably linked to the *Bt*I and *Bt*II promoters ("the *cryIAc* promoter") of the *cryIAc* gene, creating a heterologous nucleic acid operably linked to a promoter. The *cryIAc* promoter is highly active in growth conditions that induce sporulation.

## IX. EXPRESSION OF PROTEIN

**[107]** After construction and isolation of the recombinant expression vector, it is used to transform a host cell for expression of Bs binary toxin. The particular procedure used to introduce the genetic material into the host cell for expression of a protein is not particularly critical. Any of the well known procedures for introducing foreign polynucleotide sequences into host cells can be used. Transformation methods, which vary depending on the type of host cell, include electroporation; transfection employing calcium chloride, rubidium chloride calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent); and other methods (see generally Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*). In some embodiments, the host cells can be transformed by homologous recombination, as described in Poncet et al., Appl Environ Microbiol. 63:4413-4420 (1997). A preferred method of transforming *B. thuringiensis* is electroporation, as described in Wu *et al.*, *Mol. Microbiol.* 13: 965-972 (1994).

**[108]** Hosts for transformation with the Bs binary toxin gene include any suitable host bacterial cell competent to express the protein, especially members of the genus Bacillus. In particularly preferred embodiments, the cells are Bs or Bt cells. Hosts that are transformed with the Bs binary toxin are useful recombinant bacteria as insecticides. Preferred subspecies of *B. thuringiensis* include, e.g., *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *israelensis*, and *B. thuringiensis* subsp. *tenebrionis*. A preferred strain is Bti IPS82.

**[109]** After the host cell is transformed with the Bs binary toxin gene, the host cell is incubated under conditions suitable for expression of the toxin. Typically, the host will be grown under conditions that promote sporulation and expression of insecticidal endotoxin genes. Host cells may be prepared in any quantity required by fermenting an

inoculum in standard media known to those skilled in the art. The media will, for example, generally contain a nitrogen source and a carbohydrate source, e.g., glucose. Suitable conditions for incubation include a temperature in the range of 15-45°C, preferably 30°C, and an approximately neutral pH. Incubation may be conveniently carried out in batches, typically for a period of 3-5 days.

[110] Various media for growing Bt and Bs cells are known in the art. In some preferred embodiments, an inoculum from a stock host cell culture is grown on nutrient agar (BBL Microbiology Systems) or peptonized milk (1% peptonized milk [BBL Microbiology Systems], 1% dextrose, 0.2% yeast extract, 1.216 mM $MgSO_4$, 0.072 mM $FeSO_4$, 0.139 mM $ZnSO_4$, 0.118 mM $MnSO_4$) with erythromycin at a concentration of 25 μg/ml, as described in the Examples.

[111] Enhanced production of Bs binary toxin is observed after host cells competent to express the Bs binary toxin gene is transformed with the gene and the cells are grown under suitable conditions. Enhanced production of Bs binary toxin may be observed by standard methods known to those skilled in the art. For example, parasporal inclusions of insecticidal endotoxins can be purified (see Wu & Federici, *Appl. Microbiol. Biotechnol.* 42: 697-702 (1995) (hereafter "Wu and Federici 1995"), harvested by centrifugation from lysed cultures, or examined with microscopy (see Wu & Federici 1995, *supra*). Parasporal inclusions that have been harvested by centrifugation or purified may be separated using standard methods known in the art, for example, chromatography, immunoprecipitation, ELISA, bioassay, western analysis, or gel electrophoresis (see, e.g., Wu & Federici 1995, *supra*; Ausubel, *supra*). Amounts of protein are quantified by suitable means, including width and intensity of stained bands, densitometry, bioactivity, and fluorescence. For transformed Bt cells or other cells known not to synthesize Bs binary toxin in their untransformed state, all production of Bs binary toxin is considered to represent enhancement by the methods of the invention. Where Bs cells are transformed with the nucleic acids of the invention, the net amount of toxin produced by the transformed cells can be compared to like untransformed cells. Net amount of toxin refers to the amount of Bs binary toxin in parasporal bodies or crystals. The control hosts are otherwise genetically identical with the transformed hosts and grown on comparative media. Enhancement is any statistically significant increase in Bs binary toxin production. In a preferred embodiment, parasporal bodies are isolated by centrifugation from lysed cultures and are examined by SDS-PAGE gels stained with Coomassie blue.

EXAMPLES

**Example 1 Expression Levels of Bs Binary Toxin Produced in Bti Using a Bti Promoter, STAB-SD Sequence, and Coding Sequence for the Toxin**

[112] A *Bacillus sphaericus* 2362 binary toxin gene was introduced into an acrystalliferous strain (4Q7) of *Bacillus thuringiensis* subsp. *israelensis* (Bti) using *cyt1A* promoters and a STAB-SD sequence placed into the plasmid pHT3101. The construct resulted in binary toxin production which appears to be 15-fold or more greater per unit of culture medium than that obtained with the parental (wild type) *B. sphaericus* strain grown on the same medium, as assessed by densiometric scanning of gels produced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Table 1. Yield Increases Obtained Using *cyt1A* Promoters and the STAB-SD Sequence to Drive Expression of the Bs 2362 Binary Toxin Gene Operon**

| Strain | Increase in Binary Toxin | Decrease in Bti Toxins |
|---|---|---|
| Bs 2362 (wild type) | 1 | - |
| Bti IPS82 (Wild type) | - | 1 |
| Bti4Q7/Bs Binary toxin | > 15 x | |
| Bti IPS82 + Bs Binary toxin | > 20 x | .15-.35 |

**Example 2 Toxicity of Non-Toxic Bti Engineered To Express Bs Binary Toxin**

[113] The toxicity of the acrystalliferous 4Q7 Bti strain, transformed to produce Bs 2362 binary toxin, was tested on fourth instar larvae ("$L_4$") of *Culex quinquefasciatus* and compared to the wild type Bs 2362 strain grown on the same medium. (Bti strain 4Q7 does not normally produce Bs or Bti toxins.) $LC_{50}$ is the amount of toxin required to kill 50% of the larvae present in a sample during a test.

[114] As shown on Table 2, below, the amount of wild-type Bs2362 needed to kill 50% ("$LC_{50}$") of fourth instar larvae of *Culex* mosquitoes was 15.0 ng/ml. The 4Q7 Bti strain, transformed by nucleic acids of the invention to express Bs toxin, had an LC50 of 1.4, or approximately 10 times better toxicity than that of unaltered Bs.

**Example 3 Toxicity of Bti Engineered To Express Bs Binary Toxin**

[115] Transformation of *Bacillus thuringiensis* subsp. *israelensis* with the plasmid described in Example 1 that produces the Bs2362 binary toxin increased toxicity by at least 10-fold against *Culex* species compared to either of the parental strains (Bs or Bti).

[116] Bti IPS82 is the strain of Bti used as a commercial biopesticide. As can be seen from Table 2, the amount of this strain needed to kill 50% ("$LC_{50}$") of fourth instar lavae ("$L_4$") of Culex mosquitoes was 19.5 ng/ml. Wild-type Bs strain 2362 had an LC50 of 15 ng/ml. The Bti IPS82 strain, transformed by nucleic acids of the invention to express Bs toxin, had an LC50 of 1.5, or approximately 13 times better toxicity than that of unaltered Bs.

**Table 2. Toxicity of a Bti/Bs2362 Recombinant to $L_4$ *Culex quinquefasciatus***

| Strain | $LC_{50}$ (ng/ml) | Ratio <u>Bti</u> Bti/Bs | Ratio <u>Bs</u> Bti/Bs |
|---|---|---|---|
| Bti IPS82 | 19.5 | 1.0 | -- |
| Bs 2362 | 15.0 | - | 1.0 |
| Bti4Q7/Bs Binary toxin | 1.4 | - | 10.0 |
| Bti IPS82 + Bs Binary toxin | 1.5 | 13.0 | - |

**Example 4 Materials and Methods Used in Examples 1-3**

**A. Bacterial strains, gene, plasmids and transformation**

[117] Bacillus sphaericus strain 2362 was obtained from a powdered preparation that was kindly provided by Abbott Laboratories (North Chicago, IL). Escherichia coli-B. thuringiensis shuttle expression vector pHT3103 (Lereclus et al. FEMS Microbiol. Lett. 51:211-7 (1989)) was used to make and amplify the plasmid construct (pPHSP-1) in E. coli DH5α. The pPHSP-1 construct was expressed in an acrystalliferous strain, 4Q7, of B. thuringiensis subsp. israelensis obtained from the Bacillus Stock Center at Ohio State University (Columbus, OH), or in B. thuringiensis subsp. israelensis IPS82 (Abbott Laboratories). The modified pHT3101-based vector (pSTAB-SD) containing the 660-bp fragment with the cyt1A promoters and STAB-SD sequence (Agaisse and Lereclus, Mol. Microbiol., 20:633-643 (1996)) was previously described (Park et al., FEMS Microbiol Lett 181:319-327 (1999)). Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen Inc.). Bacillus strains were transformed by electroporation as described by Park et al. App Environ. Microbiol 64:3932-3938 (1998).

**B. PCR amplification of the gene encoding the *B. sphaericus* entomocidal proteins**

[118] A crude plasmid preparation was made from *B. sphaericus* 2362 using the alkaline lysis method (Sambrook *et al.*, 1989). The gene encoding the 54.1 kDa protein and 41.9 kDA entomocidal protein of *B. sphaericus* (Baumann *et al.*, J. Bacteriol. 170:2045-2050 (1988), GenBank M20390) was obtained by PCR using Vent (Exo+) DNA polymerase (Biolabs) and the primers BSP-1 (5'aactgcagCTTGTCAACATGTGAAGATTAAAGGTAACTTTCAG-3' (SEQ ID NO:10)) and BSP-2 (5'-aactgcagCCAAACAACAACAGTTTACATTCGAGTGTAAAAGTTC-3' (SEQ ID NO:11)) (Genosys). The 3.4 kbp PCR product was digested with *Pst*I and cloned in the same site in pHT3101 to generate pHBS. The 3.0 kbp *Hpa*I-*Pst*I fragment in pHBS was cloned into the filled *Xba*I and *Pst*I sites in pSTAB-SD to generate pPHSP-1.

**C. Growth of bacterial strains**

[119] The strains *B. thuringiensis* subsp. *israelensis* 4Q7/pPHSP-1 and *B. thuringiensis* subsp. *israelensis* IPS82/pPHSP-1 were grown on nutrient agar (BBL Microbiology Systems) or peptonized milk (1% peptonized milk [BBL Microbiology Systems], 1% dextrose, 0.2% yeast extract, 1.216 mM $MgSO_4$, 0.072 mM $FeSO_4$, 0.139 mM $ZnSO_4$, 0.118 mM $MnSO_4$) with erythromycin at a concentration of 25 µg/ml. For insect bioassays, *B. thuringiensis* subsp. *israelensis* IPS82/pPHSP-1 was grown in 25 ml of peptonized milk with erythromycin (25 µg/ml) in a shaker incubator set at 28 °C, 250 rpm/min for 6 days, during which time >98% of the cells had sporulated and lysed. Spores and crystals were harvested by centrifugation at 4 °C, 6,000 x g for 15 min. The -pellet was washed twice in water and dried in a vacuum chamber.

**D. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

[120] After 6 days growth in peptonized milk, 1 ml of the lysed culture was collected and centrifuged at 10,000 x g for 5 min. The medium was discarded and 150 µl of TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and 150 of 2X sample buffer (Laemmli, 1970) was added. Proteins were fractionated by SDS-PAGE (Laemmli, 1970).

**E. Bioassays**

[121] For bioassays, groups of 20 early fourth instars were exposed to a range of concentrations of the lyophilized spore/crystal powders in 100 ml of deionized water

held in 237 ml plastic cups. Seven to 9 different concentrations of the powders were replicated on 5 different days.

### F. Microscopy

[122] Sporulating cultures were monitored by light microscopy with a Zeiss Photomicroscope III, using a 100X oil immersion objective. For transmission electron microscopy, sporulated cells from peptonized milk cultures were collected just before lysis, fixed for 2 hr in 3% phosphate-buffered glutaraldehyde and 0.25% sucrose, post-fixed in 1% $OsO_4$, dehydrated in ethanol-propylene oxide, and embedded in Epon-Araldite (Ibarra and Federici, J. Bacteriol. 165:527-533 (1986)). Ultrathin sections of sporulated cells were examined and photographed in a Hitachi 600 electron microscope operating at an accelerating voltage of 75 kV.

## Example 5 Cyt1A Protein Restores Susceptibility to BS Toxin to Mosquitoes Highly Resistant to That Toxin

### A. Materials and Methods

**Bacterial strains and toxins.**

[123] Toxin preparations used in this study were lyophilized powders of lysed cultures of *B. sphaericus* 2362 and a recombinant strain of *B. thuringiensis* subsp. *israelensis* that only produces Cyt1Aa (Wu and Federici, J. Bacteriol. 175:5276-5280 (1993)). These powders contained the spore and the crystal (that is, the parasporal body) along with cell debris and media solids resulting from lyophilization. The specific powders tested were (1) *B. sphaericus* strain 2362, obtained as a technical powder of the wild-type strain from Abbott Laboratories (North Chicago, IL); (2) Cyt1Aa, a recombinant strain of BTI noted above; and (3) BTI 4Q7, an acrystalliferous strain of this subspecies that does not produce any endotoxins. This strain was obtained from the *Bacillus* Stock Center (Ohio State University, Columbus, OH) and used as one of the controls. Lyophilized powders of purified Cyt1A crystals (Wu and Federici, *supra*) were also used.

**Toxin powder production and storage.**

[124] Bacterial strains producing the various toxins were grown on solid or liquid media as described previously (Wirth et al., Proc Natl. Acad. Sci USA, 94:10536-10540 (1997), Park et al., Appl Environ Microbiol. 64:3932-3938 (1998)). The sporulated

cells were washed in distilled water, sedimented, and the resultant pellet was lyophilized. For mosquito selections and bioassays, stock suspensions of the powders were prepared in distilled water and homogenized with the aid of approximately 25 glass beads. Stocks were prepared monthly and ten-fold serial dilutions were prepared weekly. All stocks and dilutions were frozen at - 20 °C when not in use.

**Mosquito strains.**

**[125]** Two strains of *Cx. quinquefasciatus* were used; BS-R, a strain resistant to *B. sphaericus* 2362, and Syn-P, an unselected, non-resistant strain. BS-R has been selected with *B. sphaericus* 2362 since 1992 and routinely survives 48 h of exposure to 1000 μg/ml, a concentration 149,000-fold higher than the concentration which kills 50% of Syn-P, the sensitive reference strain. Syn-P is a "synthetic" population of *Cx. quinquefasciatus* derived from larval populations collected in 1995 from 3 different geographic areas in southern California. This colony has been maintained in the laboratory without exposure to *B. sphaericus*.

**Selection and bioassay procedures.**

**[126]** As noted above, the BS-R strain has been maintained under selection pressure with *B. sphaericus* 2362 since 1992. Selection consisted of exposing groups of ca. 1,000 early fourth-instars to concentrations of *B. sphaericus* ranging between 100-120 μg/ml in enameled metal pans in about 1 L of deionized water for 48-96 h. Average mortality of the larvae under selection was 10% or less per selection, and the survivors were used to continue the colony.

**[127]** For bioassays, groups of 20 early fourth instars were exposed to a range of concentrations of the lyophilized spore/crystal powders in 100 ml of deionized water held in 237 ml plastic cups. Seven to 9 different concentrations of the powders, which yielded mortality between 2 and 98% after 48 h, were replicated on 5 different days. For the bioassays in which different combinations of Cyt1A and *B. sphaericus* 2362 were tested, different ratios of these toxins were based on the weights of the lyophilized powders of the bacterial strain.

**[128]** Because the quantity of purified Cyt1A crystals was limited, bioassays with this powder utilized 10 early fourth instars held in 10 ml of deionized water in 30 ml plastic cups and replicated on 2-3 different days. Bioassays combining *B. sphaericus* 2362 technical powder and Cyt1A purified crystals at a 10:1 ratio (10 parts *B. sphaericus* 2362: 1

part Cyt1A crystal) were based on the weights of the lyophilized powders of *B. sphaericus* 2362 and Cyt1A.

[129] All data were subjected to probit analysis using a program for the PC. Dose-response values with overlapping fiducial limits were not considered to be significantly different. Resistance ratios were calculated by dividing the respective lethal concentration value for the BS-R strain by that of the Syn-P strain. Resistance ratios whose fiducial limits contained the number 1 were not considered to be significant.

**Evaluation of synergism.**

[130] Synergistic interactions between *B. sphaericus* 2362 and Cyt1A were evaluated using the method of Tabashnik, Appl Environ Entomol 58:3343-3346 (1992). Theoretical lethal concentration values for the different mixtures of Cyt1A and *B. sphaericus* 2362 were calculated from the weighted harmonic means of the individual values for these toxins. Because the *B. sphaericus* 2362 powder was not toxic to the BS-R strain at any of the concentrations tested, the calculation of the theoretical toxicity of a combination of Cyt1A and *B. sphaericus* 2362 was based on the toxicity and proportion of Cyt1A alone for this strain. The synergism factor (SF), defined as the ratio of the theoretical lethal concentration value to the observed lethal concentration value, was determined for combinations of *B. sphaericus* 2362 and the Cyt1A strain as well as for combinations of *B. sphaericus* 2362 and purified Cyt1A crystals. When the ratio was greater than 1, the toxin interaction was considered synergistic because toxicity exceeded the value predicted from individual additive toxicity. When the ratio was less than 1, the interaction was considered antagonistic, whereas a ratio of 1 indicated that the values were additive.

**B. Results**

[131] In the bioassays to determine toxin baseline values under standard conditions against the resistant and sensitive mosquito strains, no mortality resulted from exposure of BS-R, the resistant strain of *Cx. quinquefasciatus*, to 1000 μg/ml of *B. sphaericus* 2362. This concentration was 149,000 fold higher than the $LC_{50}$ (0.0067 μg/ml) obtained against Syn-P, the sensitive strain. When the bioassays were carried out in 10 ml of water with 10 larvae per cup rather than 20 larvae in 100 ml, no mortality was obtained against BS-R, but the toxicity of BS 2362 was lower ($LC_{50}$, 0.032 μg/ml) against Syn-P. Increasing larval density has been previously shown to require lower amounts of *Bti* toxin to

induce the same level of mortality observed at lower densities (Aly et al. 1988). The estimated difference in the sensitivity of BS-R and Syn-P using the smaller bioassay system was 31,000 fold.

[132] The Cyt1A bacterial strain was slightly less toxic to the BS-R strain ($LC_{50}$, 32.5 μg/ml) than to Syn-P ($LC_{50}$, 11.7 μg/ml) in the standard bioassay system. However, in the tests using Cyt1A crystals in the smaller bioassay system, no difference in sensitivity ($LC_{50}$s, ca. 20 μg/ml) was observed between BS-R and Syn-P.

[133] Adding Cyt1A to the *B. sphaericus* 2362 preparations restored most of its toxicity against the BS-R resistant *Cx. quinquefasciatus* strain. A *B. sphaericus* 2362 ratio to Cyt1A of 10:1 was highly toxic to both the resistant and sensitive mosquito strains. Toxicity levels for this combination were higher against Syn-P than BS-R, with $LC_{95}$ values of 0.442 and 36.6 μg/ml, respectively, and a resistance ratio ($LC_{95}$) of 82.9 for BS-R. The 5:1 ratio was more toxic toward Syn-P and BS-R, and the resistance ratio at the $LC_{95}$ level was reduced to 34.4-fold. At a ratio of 3:1 *B. sphaericus* 2362:Cyt1A, the mixture was again significantly more toxic to BS-R ($LC_{50}$, 1.99 μg/ml), and the resistance ratio decreased to 15.4 fold at the $LC_{95}$ level. Toxicity at a 1:1 ratio against BS-R was not significantly different from that of the 3:1 ratio. Overall, as the proportion of *B. sphaericus* 2362 to Cyt1A was increased, the toxicity increased toward both the resistant and sensitive mosquito strains. However, the resistance ratios at the $LC_{95}$ values for BS-R declined to insignificant levels for ratios of 1:3, 1:5, and 1:10, in which Cyt1A was the principal component.

[134] Calculation of the SF for these combinations revealed significant synergism between Cyt1A and *B. sphaericus* 2362 against the BS-R strain, but not against Syn-P. SF values ranged from 10 - 137 at the $LC_{95}$ level for BS-R. The highest levels of synergism were observed in the combinations in which Cyt1A was present in the lowest proportion (10:1, 5:1, 3:1). These combinations were antagonistic toward Syn-P at the $LC_{95}$ level at ratios 1:10, 1:5, and 1:3, and additive or mildly synergistic at ratios of 1:1, 3:1, 5:1, and 10:1, i.e., where *B. sphaericus* became the predominant component.

[135] Bioassays using *B. sphaericus* 2362 combined with the purified Cyt1A crystals at a ratio of 10:1 demonstrated that this combination was highly toxic to both BS-R ($LC_{95}$, 4.96 μg/ml) and Syn-P ($LC_{95}$, 2.37 μg/ml). Although the BS-R strain was slightly less sensitive to the mixture, the toxicity values were not significantly different. Importantly, no resistance was detected against the BS-R strain with this combination, which had a high SF value of 278.

### C. Discussion

[136] Combining Cyt1A with BS 2362 restored the toxicity of the latter against a highly resistant strain of *Cx. quinquefasciatus*. Moreover, we were able to completely restore toxicity with sublethal concentrations of Cyt1A crystals, and therefore suppress resistance to *B. sphaericus* in the BS-R mosquito strain. In contrast to the high level of activity observed against the resistant mosquito population, little or no enhanced activity resulted with these same mixtures against the non-resistant reference strain, Syn-P.

[137] The ability of Cyt1A at low concentrations to restore high toxicity to *B. sphaericus* 2362 against resistant mosquitoes has practical implications for control of *Culex* populations and provides insight into its mode of action. Bacterial larvicides based on *B. sphaericus* are used in several countries and resistance in field populations of *Cx. quinquefasciatus* has already been reported in France, Brazil, and India. The results of our studies indicate that adding Cyt1A at a ratio as low as 1:10 to *B. sphaericus* larvicides restores most of the toxicity against even highly resistant populations of *Cx. quinquefasciatus*. Therefore, Cyt1A provides a practical tool for managing *B. sphaericus* resistance. Furthermore, adding a small quantity of Cyt1A to *B. sphaericus* preparations can delay resistance in mosquito populations in which it has not already developed.

[138] Others have shown that a different Cyt protein, Cyt1Ab from *B. thuringiensis* subsp. *medellin*, can suppress resistance to *B. sphaericus* 2297, a mosquitocidal strain of this bacterium that produces a large toxin crystal, in *Cx. pipiens* (Thiéry et al. 1998). However, Cyt1Ab's suppression of resistance to *B. sphaericus* 2297 was much less effective than Cyt1A's suppression of resistance to *B. sphaericus*. The reduced capacity of Cyt1Ab to suppress resistance to *B. sphaericus* 2297 may be due to the 5-fold lower toxicity of this Cyt toxin to *Cx. pipiens* in comparison to Cyt1A (Thiéry et al. Appl Environ Microbiol 63:468-473 (1997)).

[139] Just how Cyt1A restores the toxicity of *B. sphaericus* 2362 is unknown. However, previous studies of the mechanism of resistance in our BS-R strain of *Cx. quinquefasciatus* and Cyt1A's binding properties suggest that Cyt1A assists binding and insertion of the toxin into the microvillar membrane. Our resistant strain of *Cx. quinquefasciatus* has no functional receptor for the *B. sphaericus* 2362 toxin and therefore it cannot bind effectively to the midgut microvilli. Studies of Cyt1A have shown that it perturbs membranes by binding to the lipid portion, and that it also binds to Cry toxins. Moreover, in the presence of the BTI Cry toxins, Cyt1A binds to the microvilli of cells in the

gastric caeca and posterior midgut of mosquito larvae. These observations suggest several mechanisms for restoring *B. sphaericus* toxicity. The Cyt1A and *B. sphaericus* toxins may bind together after dissolution, and then insert into the membrane as a complex due to Cyt1A's lipophilic properties. Another possibility is that Cyt1A may first bind to the membrane after which the *B. sphaericus* toxin binds to Cyt1A and inserts into the membrane. Finally, Cyt1A may permeate the membrane causing lesions that allow the *B. sphaericus* toxin to gain access to the original target.

**[140]** The synergism we obtained with the combinations of Cyt1A and *B. sphaericus* 2362 also provides additional evidence that Cyt1A enhances toxicity by assisting other protein toxins in binding to the mosquito microvillar membrane, especially those that do not bind efficiently. In previous studies we demonstrated that Cyt1A can synergize Cry4 and Cry11 toxins from mosquitocidal strains of *B. thuringiensis* against resistant mosquitoes. However, synergism in non-resistant mosquitoes was observed only with the Cry4 and Cry11A toxins of BTI, not with the Cry11B toxin from *B. thuringiensis* subsp. *jegathesan*; which is much more toxic than Cry11A. A similar pattern of synergism was observed in the current study wherein Cyt1A synergized the toxicity of *B. sphaericus* 2362 against the resistant BS-R strain, but not against the sensitive Syn-P strain. The implication of these results, in conjunction with those obtained in the previous studies cited above, is that toxins which are highly toxic or have a high binding affinity, such as Cry11B or the *B. sphaericus* 2362 binary toxin, gain little or no value from assisted binding by Cyt1A. But when the toxin receptors are modified or lost through resistance, Cyt1A's ability to bind to and perturb the microvillar membrane restores the capacity of these toxins to insert into the membrane and exert toxicity. As both the Cyt1A and *B. sphaericus* toxins dissolve in the mosquito midgut lumen, they may associate immediately after dissolution in the lumen as well as at the microvillar membrane surface. An implication of these results is that Cyt1A, and possibly other Cyt proteins, may extend the insecticidal spectrum of non-Cyt protein toxins to other insect species.

Table 3. Toxicity of *B. sphaericus* (strain 2362) technical powder, Cyt1A crystal/spore powder from *B. t. subsp. israelensis*, and various combinations of *B. sphaericus* and Cyt1A against susceptible (Syn-P) and *B. sphaericus* resistant (BS-R) *C. quinquefasciatus*

| Toxin(s) | Strain | No. | $LC_{50}$ (μg/ml) (fiducial limits) | $LC_{95}$ (μg/ml) (fiducial limits) | Slope (± SE) | $\chi^2$ | Resistance ratio at $LC_{50}$ (FL) | Resistance ratio at $LC_{95}$ (FL) | SF $LC_{50}$ | SF $LC_{95}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| *B. sphaericus* (strain 2362) | | | | | | | | | | |
| | Syn-P | 1,100 | 0.00671 (0.0055-0.0082) | 0.466 (0.300-0.790) | 0.89 (0.045) | 13.1 | 1.0 | 1.0 | | |
| | BS-R | 600 | No mortality at 1,000μg/ml | | | | ~149,000 | | | |
| Cyt1A | | | | | | | | | | |
| | Syn-P | 600 | 11.7 (10.2-13.4) | 59.8 (47.7-79.7) | 2.3 (0.16) | 7.3 | 1.0 | 1.0 | | |
| | BS-R | 700 | 32.5 (28.3-37.6) | 222 (172-304) | 2.0 (0.12) | 4.1 | 2.7 (2.3-3.3) | 3.7 (2.6-5.3) | | |
| *B. sphaericus* + Cyt1A (10:1)[a] | | | | | | | | | | |
| | Syn-P | 900 | 0.0288 (0.0163-0.0508) | 0.0422 (0.162-1.23) | 1.4 (0.21) | 22.8 | 1.0 | 1.0 | 0.26 | 1.2 |
| | BS-R | 800 | 2.47 (1.46-4.20) | 36.6 (14.0-97.4) | 1.4 (0.17) | 25.4 | 85.8 (56.8-129) | 82.9 (39-174) | 132 | 61 |
| *B. sphaericus* + Cyt1A (5:1) | | | | | | | | | | |
| | Syn-P | 700 | 0.0274 (0.0232-0.0322) | 0.278 (0.209-0.397) | 1.6 (0.10) | 2.4 | 1.0 | 1.0 | 0.29 | 2.0 |
| | BS-R | 1,000 | 1.23 (1.05-1.43) | 9.58 (7.49-12.9) | 1.8 (0.11) | 12.5 | 45.0 (38.1-53.2) | 34.4 (25.2-46.9) | 155.9 | 136.8 |
| *B. sphaericus* + Cyt1A (3:1) | | | | | | | | | | |
| | Syn-P | 800 | 0.0147 (0.0086-0.0354) | 0.652 (0.177-2.48) | 1.0 (0.12) | 27.1 | 1.0 | 1.0 | 0.6 | 1.0 |
| | BS-R | 600 | 1.99 (1.80-2.22) | 7.17 (5.87-9.31) | 2.9 (0.22) | 6.0 | 297 (255-347) | 15.4 (10.9-1.7) | 65 | 124 |
| *B. sphaericus* + Cyt1A (1:1) | | | | | | | | | | |
| | Syn-P | 1,000 | 0.0381 (0.0323-0.0449) | 0.464 (0.348-0.655) | 1.5 (0.08) | 10.1 | 1.0 | 1.0 | 0.35 | 2.0 |
| | BS-R | 1,000 | 0.735 (0.632-0.853) | 6.49 (5.06-8.73) | 1.7 (0.09) | 5.8 | 19.3 (16.5-22.5) | 14.0 (10.5-18.7) | 88 | 69 |
| *B. sphaericus* + Cyt1A (1:3) | | | | | | | | | | |
| | Syn-P | 900 | 0.234 (0.191-0.287) | 7.54 (5.00-12.5) | 1.1 (0.06) | 11.5 | 1.0 | 1.0 | 0.11 | 0.24 |
| | BS-R | 900 | 1.71 (1.45-2.00) | 18.4 (14.1-25.5) | 1.6 (0.09) | 6.5 | 7.3 (6.3-8.5) | 2.4 (1.8-3.2) | 25 | 16 |
| *B. sphaericus* + CytA (1:5) | | | | | | | | | | |
| | Syn-P | 1,000 | 0.189 (0.149-0.236) | 6.74 (4.66-10.6) | 1.1 (0.06) | 13.5 | 1.0 | 1.0 | 0.21 | 0.39 |
| | BS-R | 900 | 1.56 (1.34-1.81) | 11.8 (9.23-15.9) | 1.9 (0.11) | 8.9 | 8.2 (6.9-9.6) | 1.8 (1.3-2.3) | 25.3 | 23.0 |
| *B. sphaericus* + Cyt1A (1:10) | | | | | | | | | | |
| | Syn-P | 900 | 1.06 (0.859-1.29) | 25.9 (18.1-40.1) | 1.2 (0.07) | 4.8 | 1.0 | 1.0 | 0.10 | 0.17 |
| | BS-R | 900 | 4.72 (4.12-5.38) | 24.6 (19.8-32.0) | 2.3 (0.15) | 13.0 | 4.4 (3.7-5.2) | 1.0 (0.69-1.3) | 7.7 | 10.0 |

SF, synergism factor.

[a] Ratios in brackets represent the relative proportion of *B. sphaericus* technical powder to Cyt1A spore/crystal powder (BS:CytA). All ratios were based on the weight of each respective powder.

## Example 6 Use of 6-mer Polypurine Sequences and Tandem STAB-SD Sequences

[141] The ability of polypurine sequences shorter than full-length STAB-SD sequences to increase protein production was explored. As noted in the section on STAB-SD sequences, *supra*, it is believed that STAB-SD sequences in the untranslated portion of a gene enhance the production of the protein encoded by the gene by protecting mRNA from the action of 5' endoribonuclease. It is further believed that the polypurine sequences that tend to characterize STAB-SD sequences base pair with a polypyrimidine sequence at the 3' end of the 16S rRNA.

[142] Starting with the full-length 9-mer STAB-SD sequence of *cry3A*, GAAAGGAGG (SEQ ID NO:1), two 6-mer subsequences were created: AGGAGG (SEQ ID NO:12, composed of the last six nucleotides of the STAB-SD sequence), which was called PPS-I, for "polypurine sequence-I", and GAAAGG (SEQ ID NO:13, composed of the first six nucleotides of the STAB-SD sequence), which was termed PPS-III. Each PPS was placed in a construct with Bt promoters of the *cyt1A* gene, and the efficiency of each PPS as a 5' stabilizer was determined using cry3A protein as a reporter. Dual STAB-SD sequences were also tested.

[143] A series of constructs were made to test the effect on protein production of PPS-I, of PPS-III, of modifying a polypurine sequence to change some purines to pyrimidines, and of the effect of having multiple STAB-SD sequences, and the effect of separating the multiple STAB-SD sequences by different distances. All other components, (that is, the upstream region, UTR region, stem-loop structure, and coding region) were the same in all constructs.

[144] pCI-10 contained only a BtI promoter without a polypurine sequence or other stabilizing sequence. pCI-20 contained a BtI promoter and PPS-I. In pCI-21, PPS-I was modified from 5'-AGGAGG-3' to the sequence 5'-ATTATT-3', so that it no longer contained a polypurine sequence. In pCI-30, 3 upstream nucleotides of PPS-I were modified from 5'-TTT-3' to 5'-GAA-3' to return the 6-mer to the native STAB-SD sequence. Therefore, the PPS-I in pCI-20 (5'-TTTAGGAGG-3') was changed to STAB-SD in pCI-30 (5'-GAAAGGAGG-3'). pCIII-10 contained dual Bt promoters but not a PPS sequence, while pCIII-20 contained dual Bt promoters and PPS-III. pCI-50 contained two STAB-SD sequences, which were separated by 8 nucleotides. pCI-60 also contained two STAB-SD sequences, but in this construct, they were separated by 33 nucleotides.

[145] The relative amounts of Cry3A protein production of some of the constructs tested are set forth below:

pCI-10 (BtI promoter): 10%
pCI-20 (BtI promoter + PPS-I): 100% (used as a standard)
pCI-21 (BtI promoter + PPS-I without polypurine sequence ): 7%
pCIII-10 (dual Bt promoters, no PPS): 14%
pCIII-20 (dual Bt promoters + PPS-III): 37%
pCI-30 (BtI promoter + STAB-SD): 162%
pCI-50 (BtI promoter + dual STAB-SD separated by 8 nucleotides): 198%
pCI-60 (BtI promoter + dual STAB-SD separated by 33 nucleotides): 334%

[146] Thus, both PPS-I and PPS-III (contiguous 6-mers of the first 6 nucleotides of a 9-mer STAB-SD sequence and of the last 6 nucleotides from the same sequence) resulted in more protein expression than achieved from constructs without the sequences. The full length STAB-SD sequence gave markedly better results. Further, both dual-STAB-SD sequences resulted in yet higher levels of protein production, with the dual STAB-SD sequences separated by 33 nucleotide resulting in approximately double the protein production of the construct with a single STAB-SD sequence. Without wishing to be bound by theory, the different protein expression amounts exhibited by the pCI-50 and pCI-60 constructs may be due to the fact that the span length of the 30S ribosomal subunit is about 30 nucleotides. This may permit STAB-SD sequences about 30 or more nucleotides apart to interact with two 30S subunits at the same time.

[147] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[148] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A nucleic acid sequence comprising, in the following order,

(a) a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter,

(b) 6 or more contiguous nucleotides of a bacterial STAB-SD sequence,

(c) a ribosome binding site, and

(d) a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD toxin protein (SEQ ID NO:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9.

2. A nucleic acid sequence of claim 1, wherein said first polypeptide has at least 90% sequence identity to SEQ ID NO:9, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9.

3. A nucleic acid sequence of claim 1, wherein said first polypeptide has the sequence of SEQ ID NO.:9.

4. A nucleic acid sequence of claim 1, further wherein the sequence of element (d) encodes a second polypeptide with at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), which polypeptide functions as a binding domain for the 41.9 kD toxin protein of SEQ ID NO:9.

5. A nucleic acid sequence of claim 4, wherein said second polypeptide has at least 90% sequence identity to SEQ ID NO:8.

6. A nucleic acid sequence of claim 4, wherein said second polypeptide has the sequence of SEQ ID NO:8.

7. A nucleic acid sequence of claim 1, wherein said 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence.

8. A nucleic acid sequence of claim 7, wherein said 9-nucleotide bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6).

9. A nucleic acid of claim 1, wherein the *B. thuringiensis* promoter is a *cry* promoter.

10. A nucleic acid of claim 1, wherein the *B. thuringiensis* promoter is a *cry1* promoter.

11. A nucleic acid of claim 1, wherein the *B. thuringiensis* promoter is selected from the group consisting of *cry1Aa1*, *cry1Aa2*, *cry1Aa3*, *cry1Aa4*, *cry1Aa5*, *cry1Aa6*, *cry1Ba1*, *cry1Ba2*, *cry1Ca1*, *cry1Ca2*, *cry1Ca3*, *cry1Ca4*, *cry1Ca5*, *cry1Ca6*, *cry1Ca7*, *cry1Fa1*, *cry1Fa2*, *cyt1Aa1*, *cyt1Aa2*, *cyt1Aa3*, and *cyt1Aa4* .

12. A nucleic acid of claim 11, wherein the *B. thuringiensis* promoter is a *cyt1Aa1* promoter.

13. A nucleic acid of claim 1, having a BtI promoter and a BtII promoter, wherein the BtI promoter and the BtII promoter are overlapping.

14. An expression vector comprising a nucleic acid of claim 1.

15. An expression vector comprising a nucleic acid of claim 2.

16. An expression vector comprising a nucleic acid of claim 3.

17. An expression vector comprising a nucleic acid of claim 4.

18. An expression vector comprising a nucleic acid of claim 5.

19. An expression vector comprising a nucleic acid of claim 6.

20. An expression vector comprising a nucleic acid of claim 7.

21. An expression vector comprising a nucleic acid of claim 8.

22. An expression vector comprising a nucleic acid of any of claims 9 to 13.

23. A host cell comprising an expression vector of claim 14.

24. A host cell comprising an expression vector of claim 15.

25. A host cell comprising an expression vector of claim 16.

26. A host cell comprising an expression vector of claim 17.

27. A host cell comprising an expression vector of claim 18.

28. A host cell comprising an expression vector of claim 19.

29. A host cell comprising an expression vector of any of claims 20 to 22.

30. A host cell of claim 23, further comprising a *cry11A* 20 kD protein.

31. A host cell of claim 29, further comprising a *cry11A* 20 kD protein.

32. A host cell of claim 23, wherein the cell is a *B. thuringiensis* cell.

33. A host cell of claim 24, wherein the cell is a *B. thuringiensis* cell.

34. A host cell of claim 25, wherein the cell is a *B. thuringiensis* cell.

35. A host cell of claim 26, wherein the cell is a *B. thuringiensis* cell.

36. A host cell of claim 27, wherein the cell is a *B. thuringiensis* cell.

37. A host cell of any of claims 28 to 29, wherein the cell is a *B. thuringiensis* cell.

38. A nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter which binds a sigma factor A protein, at least 6 contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9.

39. A method of enhancing production of *B. sphaericus* binary toxin in a host bacterial cell, said method comprising:

(a) transforming the host cell with a nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, at least 6

contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and

(b) expressing said nucleic acid sequence in the host cell;

whereby expression of said nucleic acid sequence enhances production of *B. sphaericus* binary toxin as compared to production of *B. sphaericus* binary toxin in a wild-type *B. sphaericus* cell that is not transformed with said nucleic acid sequence.

40. A method of claim 39, wherein said first polypeptide has at least 90% sequence identity to SEQ ID NO:9.

41. A method of claim 39, wherein said first polypeptide has the sequence of SEQ ID NO.:9.

42. A method of claim 39, further wherein the sequence encoding said first polypeptide further encodes a second polypeptide with at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), which polypeptide functions as a binding domain for the 41.9 kD toxin protein of SEQ ID NO:9.

43. A method of claim 42, wherein said second polypeptide has at least 90% sequence identity to SEQ ID NO:8.

44. A method of claim 42, wherein said second polypeptide has the sequence of SEQ ID NO:8.

45. A method of claim 39, wherein said 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence.

46. A method of claim 45, wherein the bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6).

47. The method of claim 39, wherein said host cell is a *B. thuringiensis* cell.

48. The method of claim 39, wherein said host cell is a *B. sphaericus* cell.

49. The method of claim 39, wherein said host bacterial cell further expresses a 20 kD product of a *cry11A* gene.

50. A method of creating a recombinant bacterium, said method comprising the steps of:

(a) transforming the recombinant bacterium with a nucleic acid sequence comprising, in the following order:

a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter,

at least 6 contiguous nucleotides of a bacterial STAB-SD sequence,

a ribosome binding site, and

a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and

(b) expressing said nucleic acid sequence in the host cell.

51. A method of claim 50, wherein said first polypeptide has at least 90% sequence identity to SEQ ID NO:9.

52. A method of claim 50, wherein said first polypeptide has the sequence of SEQ ID NO.:9.

53. A method of claim 50, further wherein the sequence encoding said first polypeptide further comprises a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for the 41.9 kD toxin protein of SEQ ID NO:9.

54. A method of claim 53, wherein said second polypeptide has at least 90% sequence identity to SEQ ID NO:8.

55. A method of claim 53, wherein said second polypeptide has the sequence of SEQ ID NO:8.

56. A method of claim 50, wherein said 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence.

57. A method of claim 50, wherein said bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6).

58. A method of claim 50, wherein the recombinant bacterium is selected from the group consisting of *B. thuringiensis, B. sphaericus,* and a member of a *Bacillus* species other than Bti or Bs.

59. A method of increasing toxicity of a *B. thuringiensis* bacterium to a larva of a mosquito, said method comprising the steps of:

(a) transforming said bacterium with a nucleic acid sequence comprising, in the following order,

a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter,

6 or more contiguous nucleotides of a bacterial STAB-SD sequence,

a ribosome binding site, and

a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and

(b) expressing said nucleic acid sequence in the bacterium;

whereby expression of said nucleic acid sequence renders said bacterium more toxic to said larva than a wild-type *B. sphaericus* cell that is not transformed with said nucleic acid sequence.

60. A method of claim 59, wherein said bacterium further comprises a 20 kD product of the *cry11A* gene.

61. A method of claim 59, wherein said first polypeptide has at least 90% sequence identity to SEQ ID NO:8.

62. A method of claim 59, wherein said first polypeptide has the sequence of SEQ ID NO.:9.

63. A method of claim 59, further wherein the sequence encoding said first polypeptide further comprises a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for a 41.9 kD toxin protein of SEQ ID NO:9.

64. A method of claim 63, wherein said second polypeptide has at least 90% sequence identity to SEQ ID NO:8.

65. A method of claim 63, wherein said second polypeptide has the sequence of SEQ ID NO:8.

66. A method of claim 59, wherein said 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence.

67. A method of claim 66, wherein said 9-nucleotide bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6).

68. A recombinant cell of *B. sphaericus*, said cell comprising nucleic acid sequence comprising, in the following order, a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, at least 6 contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9.

69. A recombinant cell of claim 68, wherein said first polypeptide has at least 90% sequence identity to SEQ ID NO:8.

70. A recombinant cell of claim 68, wherein said first polypeptide has the sequence SEQ ID NO.:9.

71. A recombinant cell of claim 68, further wherein the sequence encoding said first polypeptide further comprises a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for a 41.9 kD toxin protein of SEQ ID NO:9.

72. A recombinant cell of claim 68, wherein said second polypeptide has at least 90% sequence identity to SEQ ID NO:8.

73. A recombinant cell of claim 68, wherein said second polypeptide has the sequence of SEQ ID NO:8.

74. A recombinant cell of claim 68, wherein said 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence.

75. A recombinant cell of claim 42, wherein said 9-nucleotide bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6).

76. A recombinant cell of claim 68, wherein the *B. thuringiensis* promoter is a *cry* promoter.

77. A recombinant cell of claim 68, wherein the *B. thuringiensis* promoter is selected from the group consisting of cry1Aa1, cry1Aa2, cry1Aa3, cry1Aa4, cry1Aa5, cry1Aa6, cry1Ba1, cry1Ba2, cry1Ca1, cry1Ca2, cry1Ca3, cry1Ca4, cry1Ca5, cry1Ca6, cry1Ca7, cry1Fa1, cry1Fa2, cyt1Aa1, cyt1Aa2, cyt1Aa3, and cyt1Aa4.

78. A recombinant cell of claim 68, wherein the *B. thuringiensis* promoter is a *cyt1Aa1* promoter.

79. A recombinant cell of claim 68, wherein said cell further expresses a 20 kD product of a *cry11A* operon.

80. A method for increasing toxicity of a *B. sphaericus* cell, said method comprising

(a) transforming the cell with a nucleic acid sequence comprising, in the

following order, a *B. thuringiensis* promoter selected from the group consisting of a BtI promoter, a BtII promoter, and a combination of a BtI and a BtII promoter, at least 6 contiguous nucleotides of a bacterial STAB-SD sequence, a ribosome binding site, and a sequence encoding a first polypeptide with at least 80% sequence identity to a 41.9 kD protein (SEQ ID NO.:9) of a *B. sphaericus* binary toxin, which first polypeptide is at least 50% as toxic as the 41.9 kD toxin protein of SEQ ID NO:9; and

(b) expressing said nucleic acid sequence in the host cell;

whereby expression of said nucleic acid sequence increases toxicity of said cell compared a wild-type *B. sphaericus* cell that is not transformed with said nucleic acid sequence.

81. A method of claim 80, wherein said first polypeptide has at least 90% sequence identity to SEQ ID NO:8.

82. A method of claim 80, wherein said first polypeptide has the sequence of SEQ ID NO.:9.

83. A method of claim 80, further wherein the sequence encoding said first polypeptide further comprises a sequence encoding a second polypeptide, which second polypeptide has at least 80% sequence identity to a 51.4 kD protein of a *B. sphaericus* binary toxin (SEQ ID NO:8), and can function as a binding domain for a 41.9 kD toxin protein of SEQ ID NO:9.

84. A method of claim 80, wherein said second polypeptide has at least 90% sequence identity to SEQ ID NO:8.

85. A method of claim 80, wherein said second polypeptide has the sequence of SEQ ID NO:8.

86. A method of claim 80, wherein said 6 or more contiguous nucleotides of a bacterial STAB-SD sequence is a 9-nucleotide bacterial STAB-SD sequence.

87. A method of claim 80, wherein said 9-nucleotide bacterial STAB-SD sequence is selected from the group consisting of GAAAGGAGG (SEQ ID NO:1), GAAGGGGGG (SEQ ID NO:2), GAGGGGGGG (SEQ ID NO:3), GAAAGGGGG (SEQ ID NO:4), GAAAGGAGG (SEQ ID NO:5), and GAAAGGGGT (SEQ ID NO:6).

88. A method of claim 80, wherein the *B. thuringiensis* promoter is a *cry* promoter.

89. A method of claim 80, wherein the *B. thuringiensis* promoter is a selected from the group consisting of *cry1Aa1, cry1Aa2, cry1Aa3, cry1Aa4, cry1Aa5, cry1Aa6, cry1Ba1, cry1Ba2, cry1Ca1, cry1Ca2, cry1Ca3, cry1Ca4, cry1Ca5, cry1Ca6, cry1Ca7, cry1Fa1*, cry1Fa2, *cyt1Aa1, cyt1Aa2, cyt1Aa3, and cyt1Aa4.*

90. The method of claim 89, wherein the *B. thuringiensis* promoter is a *cyt1Aa1* promoter.

91. A method for reducing resistance to a *B. sphaericus* binary toxin, said method comprising expressing a *B. thuringiensis* subsp. *israelensis* ("Bti") Cyt1Aa1 protein in a *B. sphaericus* cell expressing said binary toxin.

92. A method for reducing resistance to a *B. sphaericus* binary toxin, said method comprising expressing a Bti Cyt1Aa1 protein in a *B. thuringiensis* cell expressing said binary toxin

93. A method for reducing resistance to a *B. sphaericus* binary toxin, said method comprising administering Bti Cyt1Aa1 protein with said binary toxin.

94. A method of claim 93, wherein said Bti Cyt1Aa1 protein is in a powder of lysed, lyophilized Bti cells.

95. A method of claim 93, wherein said Bti Cyt1Aa1 protein is a purified protein.

96. A method of claim 93, wherein said Bti Cyt1Aa1 protein is administered in a Cyt1Aa1 protein to Bs ratio selected from about 1:2 to about 1:50.

97. A method of claim 96, wherein said Bti Cyt1Aa1 protein is administered in a Cyt1Aa1 protein to Bs ratio of about 1:10.

98. A nucleic acid of claims 1 or 38, further comprising a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence.

99. A method of any of claims 39, 50, 59 or 80, further comprising a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence.

100. A recombinant cell of claim 68, further comprising a second sequence of at least 6 contiguous nucleotides of a bacterial STAB-SD sequence.

```
        10         20         30         40         50         60
         *          *          *          *          *          *
GAATTCTATTTTCGATTTCAAATTTTCCAAACTTAAATATGATTGAATGCCTGAGAAAGG

        70         80         90        100        110        120
         *          *          *          *          *          *
TAATAGAGATGTTTTAGTTTATTATGAAGTATTAGGGGCGTCTTTTAAATTCAATCTATC

       130        140        150        160        170        180
         *          *          *          *          *          *
AATTTGTGAAATATATTACTCAAAACCCAATACCATTCTAAAACTTATTCAAAATATATA

       190        200        210        220        230        240
         *          *          *          *          *          *
                                     -35     SIGMA E   -10
TTGCTTTAAAAGAGCATACATACTAAAAAAACAGGCATCTTTCGAACTATAGCGCATAGA

       250        260        270        280        290        300
         *          *          *          *          *          *
ATACTACGGTGAATCAAAAACAAATAAAATTTAGGAGGTATATTCAAGTATACAAAAAAA

       310        320        330        340        350        360
         *          *          *          *          *          *
CTTTAGTGTGAGGGGATTTAGATAAAAAGTATTCGTTATCCTTATAAATTAATTCTTAAA

       370        380        390        400        410        420
         *          *          *          *          *          *
    -35     SIGMA K    -10
CATGCACCAATGTATACATTAAATAATATTATGTGAATTAAGTCTATCAATTTAATTTAT

       430        440        450        460        470        480
         *          *          *          *          *          *
TATGTTACTTTATATTTGATTAATAATTGCAAGTTTAAAATCATAATTTAATGTTGAAAG

       490        500        510        520        530        540
         *          *          *          *          *          *
GCCACTATTCTAATTAACTTAAGGAGTTGTTTATTTGAGCTCGGTACCCGGGGATAATct

       550        560        570        580        590        600
         *          *          *          *          *          *
 _STAB-SD_
tGAAAGGAGGgatgcctaaaaacgaagaacattaaaaacatatatttgcaccgtctaatg

       610        620        630        640        650        660
         *          *          *          *          *          *
gatttatgaaaaatcattttatcagtttgaaaattatgtattatgataagaaagtctagA

       670        680        690        700        710        720
         *          *          *          *          *          *
ACGTTATTTAATGAACTTTTTAGGTTTTAAATAATATAATGAGAAGTATTTTTTATCAAT

       730        740        750        760        770        780
         *          *          *          *          *          *
     RBS           +1
GATAAGGAGATGAAGAAAGCATGTGCGATTCAAAAGACAATTCTGGCGTTTCAGAAAAAT
                    MetCysAspSerLysAspAsnSerGlyValSerGluLys>
                  [ 51.4 kilodalton protein  >>

       790        800        810        820        830        840
         *          *          *          *          *          *
GCGGAAAGAAATTTACTAATTACCCGCTAAATACTACTCCTACAAGCCTAAATTATAACC
CysGlyLysLysPheThrAsnTyrProLeuAsnThrThrProThrSerLeuAsnTyrAsn>
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Figure 1

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      850       860       870       880       890       900
        *         *         *         *         *         *
 TTCCAGAAATATCAAAAAAATTTTATAACCTTAAGAATAAATATTCACGGAATGGTTATG
LeuProGluIleSerLysLysPheTyrAsnLeuLysAsnLysTyrSerArgAsnGlyTyr>

      910       920       930       940       950       960
        *         *         *         *         *         *
 GTTTATCAAAAACCGAATTTCCTTCAAGTATCGAAAATTGCCCATCTAACGAATATTCAA
GlyLeuSerLysThrGluPheProSerSerIleGluAsnCysProSerAsnGluTyrSer>

      970       980       990      1000      1010      1020
        *         *         *         *         *         *
 TAATGTATGATAATAAAGATCCTCGATTCTTGATTCGGTTTTTATTAGATGATGGTAGAT
IleMetTyrAspAsnLysAspProArgPheLeuIleArgPheLeuLeuAspAspGlyArg>

     1030      1040      1050      1060      1070      1080
        *         *         *         *         *         *
 ATATTATTGCAGATAGAGACGATGGAGAAGTTTTTGATGAAGCACCTACTTATTTGGATA
TyrIleIleAlaAspArgAspAspGlyGluValPheAspGluAlaProThrTyrLeuAsp>

     1090      1100      1110      1120      1130      1140
        *         *         *         *         *         *
 ATAACAATCACCCTATCATAAGTAGACATTATACCGGAGAAGAGAGACAAAAGTTTGAGC
AsnAsnAsnHisProIleIleSerArgHisTyrThrGlyGluGluArgGlnLysPheGlu>

     1150      1160      1170      1180      1190      1200
        *         *         *         *         *         *
 AGGTAGGTAGTGGAGATTATATTACGGGAGAGCAATTTTTTCAATTCTATACACAAAACA
GlnValGlySerGlyAspTyrIleThrGlyGluGlnPhePheGlnPheTyrThrGlnAsn>

     1210      1220      1230      1240      1250      1260
        *         *         *         *         *         *
 AAACACGTGTATTGTCAAATTGTAGGGCGCTTGACAGTAGGACAATATTACTATCTACTG
LysThrArgValLeuSerAsnCysArgAlaLeuAspSerArgThrIleLeuLeuSerThr>

     1270      1280      1290      1300      1310      1320
        *         *         *         *         *         *
 CAAAAATCTTCCCAATTTACCCTCCAGCTTCTGAAACTCAACTAACAGCTTTCGTTAATA
AlaLysIlePheProIleTyrProProAlaSerGluThrGlnLeuThrAlaPheValAsn>

     1330      1340      1350      1360      1370      1380
        *         *         *         *         *         *
 GTTCATTTTATGCTGCGGCAATTCCTCAATTACCCCAAACATCCTTACTTGAGAATATTC
SerSerPheTyrAlaAlaAlaIleProGlnLeuProGlnThrSerLeuLeuGluAsnIle>

     1390      1400      1410      1420      1430      1440
        *         *         *         *         *         *
 CTGAGCCTACTAGTCTCGATGATTCTGGAGTATTACCAAAAGATGCAGTAAGAGCAGTTA
ProGluProThrSerLeuAspAspSerGlyValLeuProLysAspAlaValArgAlaVal>

     1450      1460      1470      1480      1490      1500
        *         *         *         *         *         *
 AAGGAAGTGCGCTATTACCTTGTATAATAGTACATGATCCTAATTTAAACAATTCCGATA
LysGlySerAlaLeuLeuProCysIleIleValHisAspProAsnLeuAsnAsnSerAsp>

     1510      1520      1530      1540      1550      1560
        *         *         *         *         *         *
 AAATGAAATTTAATACCTACTATCTTTTAGAATATAAAGAATACTGGCATCAATTATGGT
LysMetLysPheAsnThrTyrTyrLeuLeuGluTyrLysGluTyrTrpHisGlnLeuTrp>

     1570      1580      1590      1600      1610      1620
        *         *         *         *         *         *
 CACAAATTATACCTGCTCATCAAACTGTAAAAATACAGGAACGAACAGGAATATCTGAAG
```

Figure 2

```
SerGlnIleIleProAlaHisGlnThrValLysIleGlnGluArgThrGlyIleSerGlu>

        1630      1640      1650      1660      1670      1680
           *         *         *         *         *         *
 TTGTACAAAATAGCATGATTGAAGATTTAAATATGTATATTGGAGCAGATTTTGGCATGC
ValValGlnAsnSerMetIleGluAspLeuAsnMetTyrIleGlyAlaAspPheGlyMet>

        1690      1700      1710      1720      1730      1740
           *         *         *         *         *         *
 TTTTTATTTTAGATCTAGTGGATTTAAGGAACAAATAACAAGGGGGCTAAATAGGCCTT
LeuPheTyrPheArgSerSerGlyPheLysGluGlnIleThrArgGlyLeuAsnArgPro>

        1750      1760      1770      1780      1790      1800
           *         *         *         *         *         *
 TATCCCAAACGACCACTCAGTTAGGAGAAAGAGTAGAAGAAATGGAGTATTATAATTCTA
LeuSerGlnThrThrThrGlnLeuGlyGluArgValGluGluMetGluTyrTyrAsnSer>

        1810      1820      1830      1840      1850      1860
           *         *         *         *         *         *
 ATGATTTGGATGTTAGATATGTGAAATACGCATTGGCTAGAGAATTCACACTAAAACGCG
AsnAspLeuAspValArgTyrValLysTyrAlaLeuAlaArgGluPheThrLeuLysArg>

        1870      1880      1890      1900      1910      1920
           *         *         *         *         *         *
 TTAATGGTGAAATTGTAAAAAATTGGGTTGCTGTAGATTATCGATTGGCAGGTATACAAT
ValAsnGlyGluIleValLysAsnTrpValAlaValAspTyrArgLeuAlaGlyIleGln>

        1930      1940      1950      1960      1970      1980
           *         *         *         *         *         *
 CGTATCCTAATGCACCTATAACTAATCCACTTACGCTAACAAAACATACAATTATTCGAT
SerTyrProAsnAlaProIleThrAsnProLeuThrLeuThrLysHisThrIleIleArg>

        1990      2000      2010      2020      2030      2040
           *         *         *         *         *         *
 GTGAAAATAGTTACGATGGACACATATTTAAAACACCTTTAATCTTTAAAAATGGTGAAG
CysGluAsnSerTyrAspGlyHisIlePheLysThrProLeuIlePheLysAsnGlyGlu>

        2050      2060      2070      2080      2090      2100
           *         *         *         *         *         *
 TTATTGTAAAAACGAATGAAGAATTAATACCTAAAATTAACCAGTGATACTTTAACTTCA
ValIleValLysThrAsnGluGluLeuIleProLysIleAsnGlnStop
       >  End 51.4 kilodalton protein]


        2110      2120      2130      2140      2150      2160
           *         *         *         *         *         *
 AATATTCATTACCATGTTATTTAAAATAGTAGATAGATGAAATAAATAGTATATATTAAG

        2170      2180      2190      2200      2210      2220
           *         *         *         *         *         *
 ACAACAACTTAATTTTGACACATAAGAATAATTTTTAAATGTATAAATAGTATTTAGAGT

        2230      2240      2250      2260      2270      2280
           *         *         *         *         *         *
                            RBS       +1
 GTTATTGCAATATATTTTTTGAAAGGGAGCTAAAAGACATGAGAAATTTGGATTTTATTG
                                        MetArgAsnLeuAspPheIle>
                                       [ 41.9 kilodalton

        2290      2300      2310      2320      2330      2340
           *         *         *         *         *         *
 ATTCTTTTATACCCACAGAAGGAAAGTACATTCGCGTTATGGATTTTTATAATAGCGAGT
AspSerPheIleProThrGluGlyLysTyrIleArgValMetAspPheTyrAsnSerGlu>
protein >
```

Figure 1

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    2350      2360      2370      2380      2390      2400
       *         *         *         *         *         *
 ATCCTTTCTGTATACATGCACCCTCAGCCCCTAATGGGGATATCATGACAGAAATCTGTA
TyrProPheCysIleHisAlaProSerAlaProAsnGlyAspIleMetThrGluIleCys>

    2410      2420      2430      2440      2450      2460
       *         *         *         *         *         *
 GCAGAGAAAATAATCAATATTTATTTTTTTCCTACTGATGATGGTCGAGTAATTATTG
SerArgGluAsnAsnGlnTyrPheIlePhePheProThrAspAspGlyArgValIleIle>

    2470      2480      2490      2500      2510      2520
       *         *         *         *         *         *
 CAAATAGGCATAATGGGTCCGTTTTTACCGGAGAAGCCACAAGTGTAGTATCAGATATCT
AlaAsnArgHisAsnGlySerValPheThrGlyGluAlaThrSerValValSerAspIle>

    2530      2540      2550      2560      2570      2580
       *         *         *         *         *         *
 ATACTGGTAGCCCATTACAGTTTTTTAGAGAGGTCAAAAGAACTATGGCAACTTATTATT
TyrThrGlySerProLeuGlnPhePheArgGluValLysArgThrMetAlaThrTyrTyr>

    2590      2600      2610      2620      2630      2640
       *         *         *         *         *         *
 TAGCGATACAAAATCCTGAATCCGCAACAGATGTGAGAGCTCTAGAACCGCATTCCCATG
LeuAlaIleGlnAsnProGluSerAlaThrAspValArgAlaLeuGluProHisSerHis>

    2650      2660      2670      2680      2690      2700
       *         *         *         *         *         *
 AGCTGCCATCTCGCCTTTATTACACTAACAATATTGAAAATAATAGCAACATATTAATTT
GluLeuProSerArgLeuTyrTyrThrAsnAsnIleGluAsnAsnSerAsnIleLeuIle>

    2710      2720      2730      2740      2750      2760
       *         *         *         *         *         *
 CTAATAAGGAACAAATATATTTAACCTTGCCTTCACTTCCAGAAAACGAGCAATACCCTA
SerAsnLysGluGlnIleTyrLeuThrLeuProSerLeuProGluAsnGluGlnTyrPro>

    2770      2780      2790      2800      2810      2820
       *         *         *         *         *         *
 AAACTCCAGTATTAAGCGGTATCGATGATATAGGACCTAATCAATCAGAGAAATCAATAA
LysThrProValLeuSerGlyIleAspAspIleGlyProAsnGlnSerGluLysSerIle>

    2830      2840      2850      2860      2870      2880
       *         *         *         *         *         *
 TAGGAAGTACTCTTATCCCATGTATAATGGTTTCGGATTTTATTAGTTTGGGGGAGAGAA
IleGlySerThrLeuIleProCysIleMetValSerAspPheIleSerLeuGlyGluArg>

    2890      2900      2910      2920      2930      2940
       *         *         *         *         *         *
 TGAAAACCACTCCATATTATTATGTAAAGCACACTCAATATTGGCAAAGCATGTGGTCCG
MetLysThrThrProTyrTyrTyrValLysHisThrGlnTyrTrpGlnSerMetTrpSer>

    2950      2960      2970      2980      2990      3000
       *         *         *         *         *         *
 CGCTCTTTCCACCCGGCTCTAAAGAGACAAAAACTGAGAAATCAGGTATCACTGACACTT
AlaLeuPheProProGlySerLysGluThrLysThrGluLysSerGlyIleThrAspThr>

    3010      3020      3030      3040      3050      3060
       *         *         *         *         *         *
 CTCAAATAAGTATGACTGACGGGATTAATGTTTCAATCGGAGCAGATTTCGGATTAAGGT
SerGlnIleSerMetThrAspGlyIleAsnValSerIleGlyAlaAspPheGlyLeuArg>

    3070      3080      3090      3100      3110      3120
       *         *         *         *         *         *
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Figure 1

```
TTGGAAATAAAACGTTTGGAATTAAGGGGGGGTTCACCTATGATACAAAGACTCAAATAA
PheGlyAsnLysThrPheGlyIleLysGlyGlyPheThrTyrAspThrLysThrGlnIle>

       3130      3140      3150      3160      3170      3180
          *         *         *         *         *         *
CTAATACCTCCCAATTGTTAATAGAAACAACTTATACTAGAGAATACACAAATACAGAAA
ThrAsnThrSerGlnLeuLeuIleGluThrThrTyrThrArgGluTyrThrAsnThrGlu>

       3190      3200      3210      3220      3230      3240
          *         *         *         *         *         *
ATTTTCCTGTTAGATATACAGGCTATGTTTTAGCGTCAGAATTTACTTTACATCGTAGTG
AsnPheProValArgTyrThrGlyTyrValLeuAlaSerGluPheThrLeuHisArgSer>

       3250      3260      3270      3280      3290      3300
          *         *         *         *         *         *
ATGGAACTCAGGTTAATACGATCCCATGGGTTGCTTTAAACGATAACTATACAACAATAG
AspGlyThrGlnValAsnThrIleProTrpValAlaLeuAsnAspAsnTyrThrThrIle>

       3310      3320      3330      3340      3350      3360
          *         *         *         *         *         *
CAAGATATCCACATTTTGCAAGTGAACCTTTACTAGGAAATACAAAGATTATTACAGATG
AlaArgTyrProHisPheAlaSerGluProLeuLeuGlyAsnThrLysIleIleThrAsp>
                               > end 41.9 kilodalton

       3370      3380      3390      3400      3410      3420
          *         *         *         *         *         *
ATCAAAACTAAATTTAAACAATATTCTTGAACTAATAGATGTTAAATAGAACAATTAATA
AspGlnAsnStop
 protein  ]

       3430      3440      3450      3460      3470      3480
          *         *         *         *         *         *
ACAATTTAAGTACTTTTGGATTATAGTGAAGGGACCTATAAGCATAGCTTTTAGGTCCCT

       3490      3500      3510      3520      3530      3540
          *         *         *         *         *         *
TTTAAGTTGCTTTTTTTCGTTTTTAGAATAGTATAGATAGGCTACACTACACTAAGTTGG

       3550      3560      3570      3580      3590      3600
          *         *         *         *         *         *
ACAGATAAAATAAGGGGTTGTAAACTTAGACTATTAAAAAAGGGAGAGTGCTACTATGAC

       3610      3620      3630      3640      3650
          *         *         *         *         *
ACGTCAACATCGAACTTTTACACTCGAATGTAAACTGTTGTTGTTTGGCTGCAG
```

Figure 1

US 100747820AP1

Creation date: 11-13-2003
Indexing Officer: DSHEA - DUNCAN SHEA
Team: OIPEBackFileIndexing
Dossier: 10074782

Legal Date: 03-11-2003

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